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14. ABSTRACT
Neuropathy target esterase (NTE) is a membrane-associated protein with serine esterase activity. A class of organophosphate (OP) compounds, used in insecticides and as chemical weapons, are capable of inhibiting NTE and lead to progressive neuropathies. We were able to isolate and characterize the human and mouse NTE (mNTE) genomic loci. We also identified a second member of the NTE family. Transgenic mice with a disrupted mNTE gene and which express the β-galactosidase gene under the endogenous mNTE promoter were generated. Analyses demonstrated that mNTE is essential for emobryonic development and that mNTE is highly expressed in the developing spinal cord and eye, as well as in the testes and throughout the brain. Heterozygous mice have a 39% decrease in brain enzyme activity and increased mortality after exposure to NTE-inhibing EOPFs. Wid-type mice treated with low amount of EOPF and untreated mNTE heterozygous mice show elevated motor activity, suggesting that partial inhibition of NTE activity leads to hyperactivity. Further analysis of these mice also allowed us to identify NTE as a lysophospholipase.

15. SUBJECT TERMS
Neurodegeneration, organophosphorus, neutotoxicity, genetic, Esterase, transgenic, mouse, human

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INTRODUCTION

Organophosphorus esters (OP) are among the best known neurotoxins (Chambers and Levi, 1992). These compounds act predominantly at cholinergic synapses by inhibiting acetylcholinesterases and producing acute toxicity. However, there are other OP toxicities which are delayed (Cavanagh, 1973; Haley and Kurt, 1997; Johnson, 1975; Johnson, 1990; Johnson and Glynn, 1995; Karczmar, 1984) (and for a recent review (Jamal, 1997)). The most common and well studied delayed syndrome due to OP toxicity is known as OP-induced delayed neuropathy (OPIDN) and is believed to involve inhibition of an esterase other than acetylcholinesterase (Johnson, 1969). A neuronal protein fraction with esterase activity was identified by several groups and was believed to be the target for the initiation of delayed neuropathy induced by some OP's (Chambers and Levi, 1992; Johnson, 1990; Johnson and Glynn, 1995). This protein is Neuropathy Target Esterase (NTE). NTE is targeted specifically by OPs that cause OPIDN (for review (Johnson and Glynn, 1995) and references therein). It is believed that long term repeated exposure to NTE inhibitors can lead to permanent chronic neuropsychopathological disease affecting behavior as well as cognitive and visual functions (Karczmar, 1984) (for review see(Jamal, 1997)). Importantly, it is also postulated that neurotoxic chemical combinations, such as pesticides and insect repellents, which inhibit NTE in conjunction, but not in isolation, may cause more severe brain dysfunction (Haley and Kurt, 1997; Wilson et al., 2002). Several important questions remain unanswered and are of critical importance in establishing the precise role of NTE and consequences of its inhibition in a living organism. Most importantly, the physiologic function of NTE is unknown. Recent evidence has suggested that the NTE catalytic esterase (NEST) domain is capable of hydrolysis of membrane associated lipids, and may suggest a function for NTE in membrane trafficking and cell signaling (Atkins et al., 2002; van Tienhoven et al., 2002). However, it is entirely unclear what the natural substrate for NTE activity is or whether the protein actually has other, as yet unrealized functions that are important for neuronal survival. Our aim was to elucidate the normal role of NTE in a mammalian organism using a combined molecular, genetic and cell biological approach.

BODY OF SUMMARY STATEMENT

The original statement of work aimed to map human and mouse NTE, perform expression analysis of NTE, produce a mouse model deficient in mNTE and develop a cell culture system for the study of NTE. In the prior reports we described this work in detail. In addition, our work was recently published in the journal Nature Genetics. Several editorials regarding this work have been published including a News and Views in Nature Genetics, and a review in Lancet (see appendix items for details). More recently we published a paper in PNAS where we have used the mouse model to understand a new target for NTE (Quistad et al., PNAS 2003-Appendix 3). In this work we show that NTE has lysophophospholipase activity. Therefore, OP-induced delayed toxicity in mice may be due to NTE-Lysophospholipase inhibition leading to localized accumulation of lysolecithin, a known demylelinating agent and re receptor-mediated signal transducer. I will not provide a specific progress report with regard to this work, rather I have attached the appropriate publications (Winrow, Nature Genetics 2003-Appendix 2 and Quistad et al, PNAS 2003-Appendix 3).

We have been unable to generate the conditional knockout. However, while we were attempting to do this, another group published their findings. Importantly, this group showed that the complete absence of NTE in the developing brain resulted in severe and fatal brain disorders. Taken together, these pivotal studies conclusively show that NTE is essential for the normal

function of the brain and that the mechanism whereby NTE is essential for the nervous system is through its role as a lysophospholipase.

Finally, an important component of our work is to ensure that the reagents generated as part of these efforts are available for other researchers to use. We applied to the Jackson Laboratories for acceptance of our mice and for them to act as a repository for the animals. Jackson Laboratories accepts a very small number of mice into the repository for distribution and rigorously evaluates the benefits and impact of each application. Jackson Laboratories recognized the importance of our mice and has agreed to accept the mice. We have worked with them and the mice are currently in the process of transfer (please see attached Appendix 8 and 9). In addition, investigators have directly expressed interest in collaborations to further characterize the NTE deficient mice. One of the interested investigators is Dr. Robert Kan (USAMRICD, Aberdeen Proving Ground, Maryland) who has suggested examining the effects of nerve agents such as Soman and AChE inhibitors on the behavior and pathology of the NTE deficient mice. We are currently following up with Dr. Kan.

Generation of a conditional NTE mutant

In our original proposal we had also planned to generate a targeting construct that could be used for tissue specific knockouts in the event that complete loss of NTE might be embryonic lethal (the conditional knockout). Over the past year we were able to generate a targeting construct, transfect ES cells and isolate neomycin resistant colonies. We were also able to identify two correctly targeted clones (**Figure 1**). We had hoped to have already generated a mouse for study this year but the work-load for the characterization of the heterozygous mice has not allowed us time to generate conditional mice. We have also successfully generated several different CRE expressing mice for use with conditional knockout. We have attempted to generate mice from these clones and have been unsuccessful so far. We plan to go back to other clones and work through the issues again. We have completely characterized the appropriate CRE mice so anticipate that once we get the germline transmission we will be able to rapidly produce the appropriate animals. In the interim, we continue to use the NTE+/- mice for our work and they are proving very useful in understanding how OP result in toxicity (see attached PNAS paper).

Unfortunately, none of the clones had germline transmission and given that another group of investigators has successfully made the animals, we will no longer pursue this goal, rather encourage the investigators to similarly apply to the Jackson Laboratories to deposit their mice for other investigators to use.

KEY RESEARCH ACCOMPLISHMENTS

We report for the first time the mapping and characterization of the mouse NTE locus and describe the generation of transgenic mice lacking one or both alleles of mNTE. We used the powerful technique of manipulating the mouse genome to create a mammalian model which lacks NTE and use this model to study the normal physiologic role of the protein as well as its role in OP-induced neurotoxicity. We show that unlike in Drosophila, mNTE is essential for embryonic survival in mice. Further we show that mNTE heterozygous mice have increased motor activity, reduced NTE esterase activity in the brain and are more sensitive to OP-induced toxicity. This is the first reported model system for studying the effects of reduced NTE activity both genetically and chemically in mammals, and provides a much needed reagent for carrying out analyses of OPIDN, as well as for studying it's role in normal neuronal development, maintainence and neurodegeneration.

The characterization of the mouse and human NTE genes provides an enormous step forward for us to dissect the function of NTE and its role in OP induced toxicities. A major

shortcoming of models where OPs are administered to mammals is their inability to mechanistically implicate the role for NTE in the neurotoxicity since the OPs used may have other targets unrelated to NTE and the association with NTE inhibition may be an epiphenomenon for some of the effects. Our work conclusively identifies NTE as a target of this class of OP's. We have been successful in identifying and generating reagents which will allow us to determine how OPs exert their effects in the mammalian brain. Understanding the mechanism of action of NTE will allow us to understand why binding of OP's to NTE result in brain disease. This is an important step in determining how best to protect the brain from the toxicity of these compounds. The production of a mammalian organism with a defined genetic mutation may serve as an entry point to investigate pathways critical for normal brain function. In addition, this type of investigation will lead to a greater understanding of this and other neurodegenerative disease, provide novel therapeutic approaches, and provide animal models to test therapies.

Figure Legends

Figure 1 - Generation of ES cells for producing a conditional mNTE Knock-out mouse.
(A) Schematic representation of the strategy for creating a conditional disruption of mNTE using the Cre-LoxP system. The wild-type (WT) locus is shown at the top of the panel, with the targeting vector underneath. The resulting recombined loci are shown below. LoxP sites are indicated in green, flanking the neomycin resistance gene and exons within the mNTE locus. Following initial selection of ES cells containing the correctly recombined locus, the ES cell clones will be exposed to Cre recombinase and those clones which lose the NeoR gene will be isolated and used for the generation of transgenic animals. The result of LoxP recombination in mNTE-LoxP mice will be the deletion of a region of the mNTE locus as shown. (B) Southern blot screen of ES cells using a probe, which will hybridize to a 10.4 kbp WT band and a 4.2 kbp correctly targeted band. Potentially positive clones are indicated and will be subsequently validated by PCR and further Southern analyses.

REPORTABLE OUTCOMES

- Manuscripts: Nature Genetics and PNAS manuscripts with associated reviews of the data are attached.
- Abstracts: none
- Presentations: **Winrow, C.J.** (2004) Loss of Neuropathy Target Esterase in mice links organophosphate exposure to hyperactivity. Invited speaker at 2004 International Meeting for Autism Research – Autism, Genes and Environment Session. Sacramento, California.
- Patents and licenses applied for and/or issued:

  U.S. Serial No. 60/379,937, filed May 10, 2002, entitled **A neuropathy Target Esterase (nte)-deficient Mouse Model and Method for the Treatment of Peripheral Neuropathies**. Inventors: Barlow and Winrow
- Degrees obtained that are supported by this award: Honors thesis project for Mathew Hemming
- Cell lines: none
- Informatics: none

- Animal models: see above – deposited to Jackson Laboratories, Appendix 8.
- Funding applied for based on this work: none
- Employment or research opportunities applied for and/or received on experience/training supported by this award: Duane Allen, who joined the project as my first technician and who has completed the majority of this work was recently accepted in the neuroscience graduate program at the University of California, San Francisco. Amber Pope, who also contributed to the project was also accepted to Harvard as an undergraduate and completed her first year last year. Chris Winrow recently joined Merck Research Lab as a Senior Research Biologist. Mathew Hemming is completing his honor's thesis project for his undergraduate degree in Biology at UCSD and was recently accepted into Harvard's Neuroscience Graduate Program.

CONCLUSIONS

This work demonstrated for the first time that mammalian NTE is essential for normal development. Because of its critical role in development, loss of NTE resulted in embryonic lethality. Therefore, we were unable to identify the consequences of complete NTE loss of function in the nervous system. Although we were able to generate embryonic stem cell clones and the appropriate CRE transgenic mice for further experiments to address the role of NTE in the brain (by overcoming embryonic lethality), we were unable to complete the experiments in the short time frame of this grant. In addition, the current funding will help us maintain and breed these animals for distrubution to various labs who have requested the mice. We will also complete the work to transfer the mice to Jackson Laboratories for maintainence and distribution.

Regardless, we were able to show that even partial inhibition of NTE results in a clear neurobehavioral phenotype of hyperactivity. Future studies will need to be pursued to ascertain what other effects partial loss of NTE function has on the nervous system. Importantly, this study conclusively shows that chemical inhibition of NTE by OP's mimics the phenotype caused by partial loss of NTE through genetic haploinsufficiency. Therefore, we conclusively show for the first time that OP's that cause neurological sequelae act through inhibition of NTE and this inhibition is detrimental to the nervous system of mammals. The NTE haploinsufficient mouse model will be an extremely useful reagent for understanding how nerve gas poisons and pesticides act in combination to cause neurological dysfunction in man.

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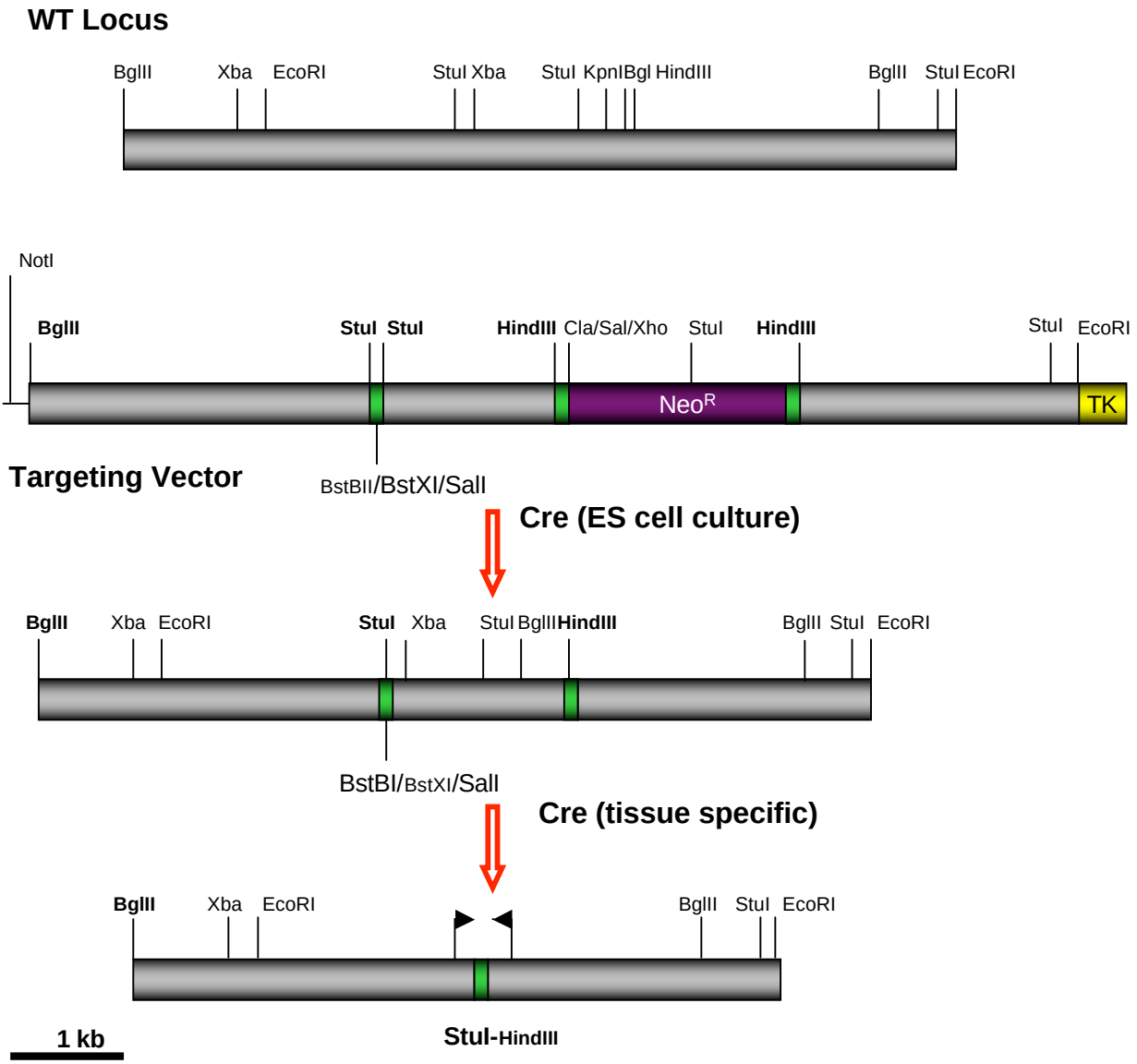
APPENDICES

Appendix 1: Figure 1
Appendix 2: Winrow et al, Nature Genetics 2003
Appendix 3: Quistad et al., PNAS 2003
Appendix 4: News and views from Nature Genetics
Appendix 5: Correspondence from Nature Genetics
Appendix 6: Lancet correspondence
Appendix 7: Science news correspondence
Appendix 8: Jackson Laboratory NTE stock inventory information
Appendix 9: Jackson Laboratory NTE genotyping protocol

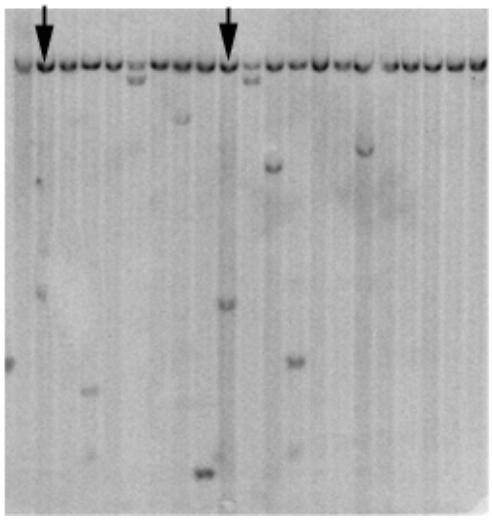
Figure 1

Conditional KO Overview

A.



B.



Loss of neuropathy target esterase in mice links organophosphate exposure to hyperactivity

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Neuropathy target esterase (NTE) is involved in neural development and is the target for neurodegeneration induced by selected organophosphorus pesticides and chemical warfare agents. We generated mice with disruptions in *Nte*, the gene encoding NTE. *Nte*$^{-/-}$ mice die after embryonic day 8, and *Nte*$^{+/-}$ mice have lower activity of Nte in the brain and higher mortality when exposed to the Nte-inhibiting compound ethyl octylphosphonofluoridate (EOPF) than do wild-type mice. *Nte*$^{+/-}$ and wild-type mice treated with 1 mg per kg of body weight of EOPF have elevated motor activity, showing that even minor reduction of Nte activity leads to hyperactivity. These studies show that genetic or chemical reduction of Nte activity results in a neurological phenotype of hyperactivity in mammals and indicate that EOPF toxicity occurs directly through inhibition of Nte without the requirement for Nte gain of function or aging.

Introduction

The acute toxic effects of organophosphate insecticides and chemical warfare agents are due to inhibition of acetylcholinesterase[1,2]. There are also delayed effects for a subset of organophosphates. In humans, delayed chronic neurotoxic syndromes from exposure to individual organophosphates or combinations of agents have been reported for Gulf War veterans and chronically exposed individuals[3–9]. The most common and best understood delayed syndrome is organophosphate-induced delayed neuropathy (OPIDN). Current knowledge of OPIDN is based on studies of more than 30,000 human cases and the use of hens as the preferred model. OPIDN is characterized by paralysis of the lower limbs due to degeneration of long axons in the spinal cord and in peripheral nerves. The proposed target protein is NTE[10,11].

The multistep hypothesis of organophosphate toxicity is as follows: (i) organophosphate toxicants selectively inhibit NTE relative to acetylcholinesterase; (ii) neuropathic effects are observed only after NTE activity is inhibited by 70–90% (refs. 11,12); (iii) NTE is phosphorylated at the serine residue in the catalytic site; (iv) loss of an alkoxy group (referred to as aging) leaves a negatively charged phosphate at the active site; (v) a toxic gain of function leads to neurodegeneration[10–13]. But the organophosphates used to establish the multistep hypothesis may have multiple targets, and the association with NTE inhibition may be an epiphenomenon. Elucidation of the primary structure of human NTE and mouse Nte did not in itself suggest a physiologic function but did establish homology to the *Drosophila melanogaster* swiss cheese (SWS) protein[14,15]. SWS is not essential for embryonic survival, but flies lacking the *sws* gene undergo glial hyperwrapping and subsequent neurodegeneration followed by death[16]. These are the only known consequences of reducing levels of SWS and, by analogy, NTE in an organism. Therefore, it is important to define the role of NTE in mammals and the consequences of its inhibition by organophosphates.

Characterization of the mouse and human genes encoding NTE (*Nte* and *NTE*, respectively) is a key step in dissecting the function of NTE and its role in organophosphate-induced toxicities. The mouse is a useful model for studying the effects of organophosphates[17–19], even though it has a different spectrum of neuropathology and toxicity than does the hen[11,12,20,21]. Organophosphate-induced sub-acute neurotoxicity in mice is similar to OPIDN in hens in the correlation with NTE inhibition and the prophylactic actions of reversible NTE inhibitors but differs from OPIDN in the time of onset of toxicity and a greater incidence of fatality[19,21,22].

Here we report the mapping and characterization of the *NTE* and *Nte* genomic loci and describe the generation of mice lacking *Nte*. Unlike *sws* in *D. melanogaster*, *Nte* is essential for embryonic survival. In addition, we found that mice heterozygous with respect to the *Nte* mutation (*Nte*$^{+/-}$) had lower Nte activity in the brain, greater motor activity and greater sensitivity to EOPF. Our study shows that it is the reduction of NTE activity and not a gain-of-function phenotype that is responsible for the toxic effects of organophosphate agents. This is the first system for studying the effects of reduced NTE levels in mammals and provides a much-needed model system for carrying out analyses of potential clinical syndromes caused by organophosphate exposure.

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Results

Characterization of *NTE* and *Nte* genomic loci

To study the *Nte* genomic locus, we sequenced a mouse expressed-sequence tag (EST) clone homologous to the 5′ end of *NTE* and used it to design primers to screen a 129S6/SvEvTac (129S6) mouse bacterial artificial chromosome (BAC) library (Incyte Genomics, Down-to-the-Well platform). This screen identified BAC clone 59B13. We sequenced this clone throughout the *Nte* locus and used it as a probe for fluorescence *in situ* hybridization (FISH) of mouse metaphase chromosome spreads (Fig. 1*a*). We also identified a human BAC clone (48M5) and used it for FISH of human metaphase chromosomes (Fig. 1*a*). FISH showed that the *NTE* loci mapped to human chromosome 19p13.3 and mouse chromosome 8A1.1, consistent with data from the National Center for Biotechnology Information (NCBI). A high level of conservation is observed between the *NTE* and *Nte* genomic loci (Fig. 1*b*). Both loci span 35 exons, over 27 kb in human and 29 kb in mouse, and intron-exon boundaries are highly conserved (Fig. 1*b*). By combining standard library screening with database searches, we also identified an *Nte* liver splice variant (Fig. 1*b*). This alternate transcript contains 14 exons, is 1659 bp in length, uses an exon not present in the predominant form of Nte and lacks the Nte esterase (NEST) domain and two of three cyclic nucleotide monophosphate (cNMP)–binding domains[23].

Identification of an *NTE*-related gene

In silico analysis of public and commercial databases also identified a related *NTE* locus termed NTE-related 1 (*NTE-R1*). We identified partial clones in the GenBank database and the full genomic locus using the Celera Discovery System. The *NTE-R1* locus is conserved in mouse, rat and human (Fig. 1*c*). The human *NTE-R1* gene is localized to chromosome 9 and spans 31 exons over 90.8 kb. The mouse *Nte-R1* is localized to chromosome 2 and spans 27 exons over 74.7 kb. We identified several ESTs matching the *NTE-R1* transcript in human and mouse in nine tissues, indicating that the *NTE-R1* mRNA is present in multiple locations. The predicted transcript is 3305 bp in length in mouse and 4522 bp in humans. There is a high degree of homology to *NTE* at the nucleotide (76% identity in humans and 74% identity in mice) and amino-acid levels, with 65% homology (55% identity) in humans and 73% homology (62% identity) in mice. The catalytic NEST/patatin domain[23,24] (including the catalytic residues) is retained in NTE-R1 (Fig. 1*c*) and is highly conserved with 82% homology (68% identity) in human and 91% homology (75% identity) in mouse (Fig. 1*c*).

Generation of mice deficient in Nte

We generated a restriction map of the 129S6 BAC clone 59B13 and used a genomic fragment of 15.2 kb to construct the *Nte* targeting vector (Fig. 2*a*). The targeting vector was designed to result in an in-frame fusion between exon 4 of *Nte* and the *lacZ* gene (lacking a start ATG and promoter) and to delete exons 5–10 by inserting the neomycin-resistance gene (*neo*^{r}) followed by multiple stop codons. With correct recombination a full-length

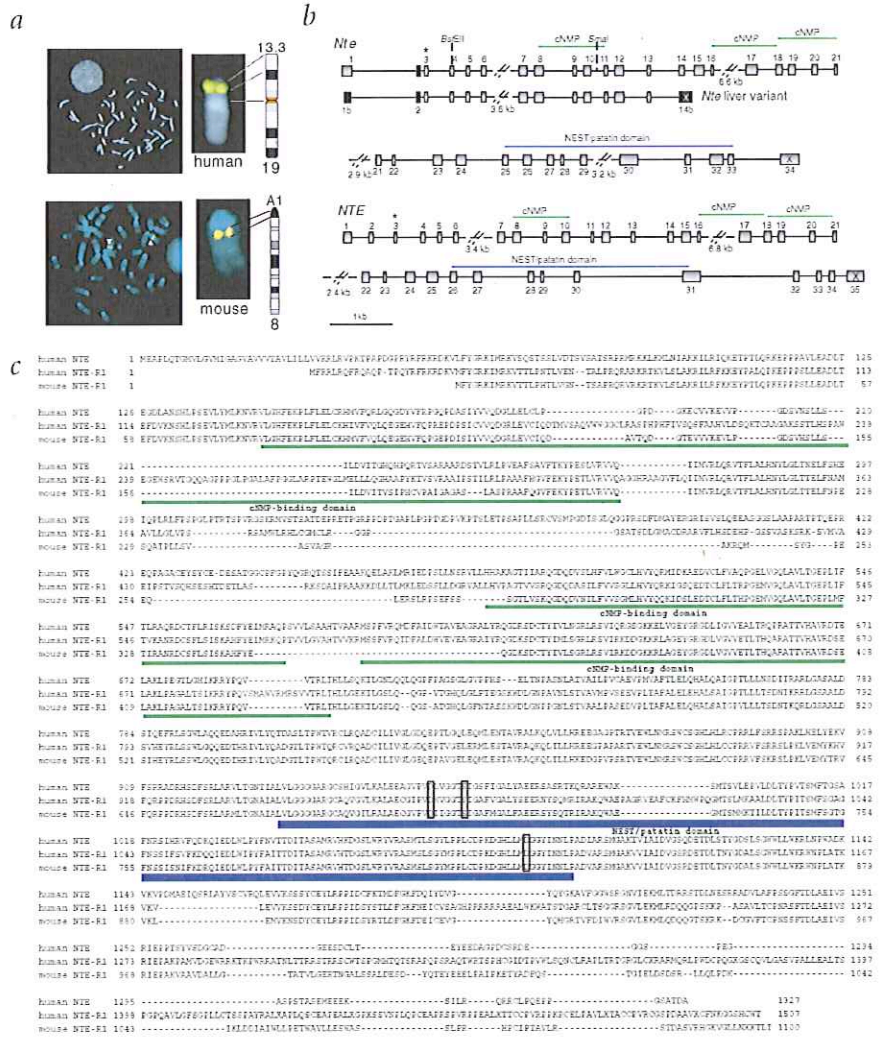


Fig. 1 Characterization of the *NTE* and *Nte* genomic loci and identification of *NTE-R1*. *a*, FISH analysis was done on human metaphase chromosomes (upper panel) using the BAC clone 48M5 and on mouse metaphase chromosomes (lower panel) using the BAC clone 59B13. *NTE* is localized to chromosome 19p13.3 and *Nte* to chromosome 8A1.1. *b*, Schematic of the *NTE* loci in mouse (top) and human (bottom) and the alternatively spliced *Nte* transcript isolated from liver (*NTE* liver variant). Exons are shown as gray blocks, and those containing the stop codon are indicated with an "X". The exon containing the start ATG is indicated by an asterisk and solid black blocks indicate alternatively spliced exons found in the *Nte* liver variant. The conserved cNMP-binding domains and the NEST/patatin domain are indicated by green and blue lines, respectively. The *Bst*EII and *Sma*I sites used for constructing the *Nte* targeting vector are shown. The schematic also shows the high level of intron/exon conservation between the *NTE* and *Nte* loci. *c*, Clustal alignment of human NTE and the human NTE-R1 and mouse Nte-R1 proteins. The three cNMP-binding domains (green line) and the NEST/patatin domain (blue line) are conserved among all three proteins. Amino acids that form the catalytic triad within the NEST/patatin domain are also conserved and are shown in boxes.

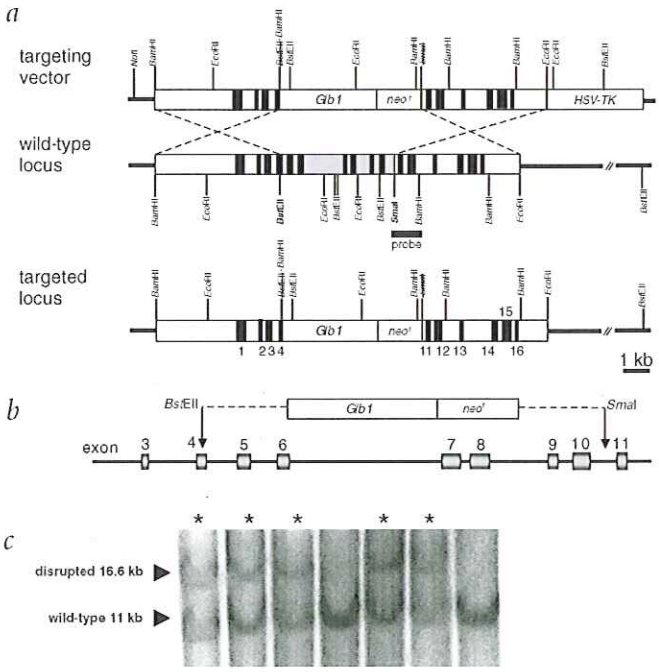
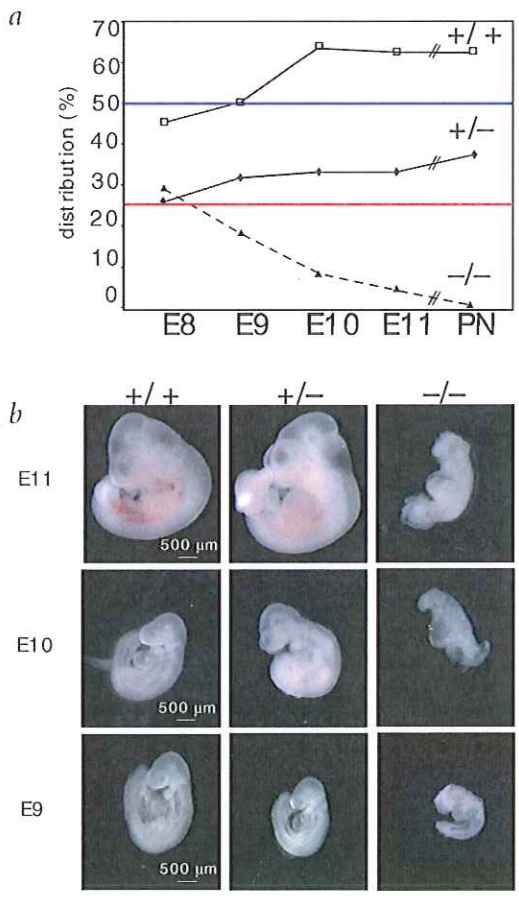


Fig. 2 Generation of mice with disruptions in *Nte*. **a**, The *Nte–lacZ* targeting vector is shown above the normal $Nte^{+/+}$ locus and the targeted locus. Exons are indicated as gray blocks with exon numbers given below the targeted locus. β-galactosidase (*Glb1*), neomycin resistance (neo^r) and thymidine kinase (*HSV-TK*) genes are shown and a probe used for Southern-blot screening is indicated as a horizontal bar. Correct integration results in the elimination of *Bst*EII and *Sma*I sites flanking the genes *Glb1* and neo^r (indicated by strikethroughs). **b**, The correctly targeted locus results in the in-frame fusion of the *lacZ* gene to *Nte* exon 4 at a *Bst*EII site, deletion of exons 5–10 and insertion of the neo^r gene at a *Sma*I site. The *lacZ* and neo^r genes contain stop codons and polyA sites, and the downstream exons are out of frame from exons 1 and 2, thereby decreasing the likelihood of splicing around the targeted insertion. **c**, Southern blot of genomic DNA from F1 mice, digested with *Bst*EII and hybridized with the probe shown in **a**, showing correct integration of the disrupted Nte^r allele in the $Nte^{+/-}$ mice (lanes with an asterisk).

Nte transcript would not be produced. Expression of *lacZ* from the endogenous *Nte* promoter (Fig. 2*b*) allowed for the visualization of β-galactosidase activity in the mutant mice, corresponding to the normal pattern of *Nte* expression. The overall result of this strategy was the elimination of a functional *Nte* transcript.

We isolated a set of 114 neomycin-resistant embryonic stem cell (ES) colonies. Nine ES clones were correctly targeted and four independent ES clones were used to generate chimeras. Two of the chimeras from independent ES clones showed high-level germline transmission. We mated the chimeras to obtain offspring carrying the mutated allele. Southern-blot analysis of tail samples identified mice with a properly targeted *Nte* allele (Fig. 2*c*). Both independent ES clones gave rise to mouse lines with identical phenotypes.



Nte is essential for embryonic survival

Northern-blot analysis showed that *Nte* was expressed as early as embryonic day 7 (E7) and throughout embryonic development (data not shown). Loss of both *Nte* alleles resulted in lethality evident at E9 (Fig. 3). The expected genotype distribution of mutant, wild-type and heterozygous mice was observed at E8 (Fig. 3*a*), but the percentage of $Nte^{-/-}$ embryos decreased after E8, and no $Nte^{-/-}$ embryos were found after E11 (Fig. 3*a*). $Nte^{-/-}$ embryos isolated at E9 had aberrant morphology and signs of resorption (Fig. 3*b*). This shows that Nte is essential for embryonic survival beyond E8. The cause of lethality is not apparent, but it may be due to defective closing of the neural tube. In contrast, the $Nte^{+/-}$ mice were viable and fertile.

Nte expression patterns

We stained $Nte^{+/-}$ mice at various ages for β-galactosidase activity to identify the normal patterns of *Nte* expression (Fig. 4). We observed high levels of expression in restricted patterns throughout the $Nte^{+/-}$ adult mice and embryos. In particular, $Nte^{+/-}$ embryos at E13.5 showed strong expression in the cells of the

Fig. 3 $Nte^{-/-}$ mice are not viable beyond E8. **a**, Changes in the genotype ratios (percentage distribution) during embryonic development showed that Nte is essential for embryo survival. +/+ indicates wild-type, +/– indicates heterozygote and –/– indicates homozygote with respect to the mutated *Nte* allele. Total number of embryos were $n = 31$ at E8, $n = 22$ at E9, $n = 36$ at E10, $n = 24$ at E11 and $n = 144$ post-natal (PN). At E8 the embryo distribution was close to the expected genotype ratio of 50% heterozygotes (blue line), 25% wild-type and 25% homozygotes (red line). But the percentage of $Nte^{-/-}$ embryos decreased through development and no $Nte^{-/-}$ mice were observed postnatally. **b**, Photomicrographs of embryos isolated at E11, E10 and E9 with corresponding genotypes. E9 $Nte^{-/-}$ embryos seemed developmentally delayed and runted compared with age-matched $Nte^{+/-}$ and wild-type embryos. $Nte^{-/-}$ embryos at E10 and E11 seemed arrested in development, were much smaller than wild-type and $Nte^{+/-}$ embryos and showed signs of resorption. These observations suggest that $Nte^{-/-}$ embryos do not develop properly beyond E8.

developing lens (Fig. 4*b–f*) and along the developing spinal cord (Fig. 4*a*,*b*,*g–j*). In the adult, expression was particularly robust in the Leydig cells of the testes, although faint staining was also observed in the testes of wild-type mice (Fig. 4*k–n*). Expression was observed throughout the brain, particularly in the cortex, in the Purkinje cells of the cerebellum (Fig. 4*o–v*) and in the hippocampus (Fig. 4*s–v*). These expression patterns are in agreement with northern-blot analysis (data not shown) and previous *in situ* hybridization experiments[15].

Nte$^{+/-}$ mice have lower activity of Nte but not acetylcholinesterase

To determine the effect of disrupting one allele of *Nte*, we assessed the activities of Nte and acetylcholinesterase. We observed approximately 40% lower enzymatic activity of Nte in the brain in *Nte*$^{+/-}$ mice compared with wild-type mice (Fig. 5*a*; $P < 0.0001$). A somewhat smaller reduction in Nte activity was observed in testes (Fig. 5*a*). This effect was specific to *Nte*, as there was no difference in the level of acetylcholinesterase activity between wild-type and *Nte*$^{+/-}$ mice (Fig. 5*a*). The level of Nte protein was also lower in the brain, testes and kidney (Fig. 5*b*) but not in the liver (data not shown), as determined by immunoprecipitation and western blotting with a mouse-specific Nte antibody (Fig. 5*b*). Although the *Nte*$^{+/-}$ mice had lower Nte protein levels and activity, there were no signs of neuropathy or other obvious pathology.

Nte$^{+/-}$ mice are more sensitive to organophosphate-induced toxicity

To ascertain the role of Nte in organophosphate-induced toxicity, we tested whether *Nte*$^{+/-}$ mice were more or less sensitive than wild-type mice to an organophosphate compound that causes delayed toxicity. *Nte*$^{+/-}$ mice and wild-type littermates were injected intraperitoneally with 6 and 10 mg EOPF per kg of body weight. The high potency of EOPF as an Nte inhibitor *in vitro* in mouse and hen brain (IC_{50} values of 0.02–0.04 nM) and *in vivo* in mouse brain (85% inhibition at 5 mg per kg body weight; refs. 19,25) makes it an optimal compound for studies in mice. We

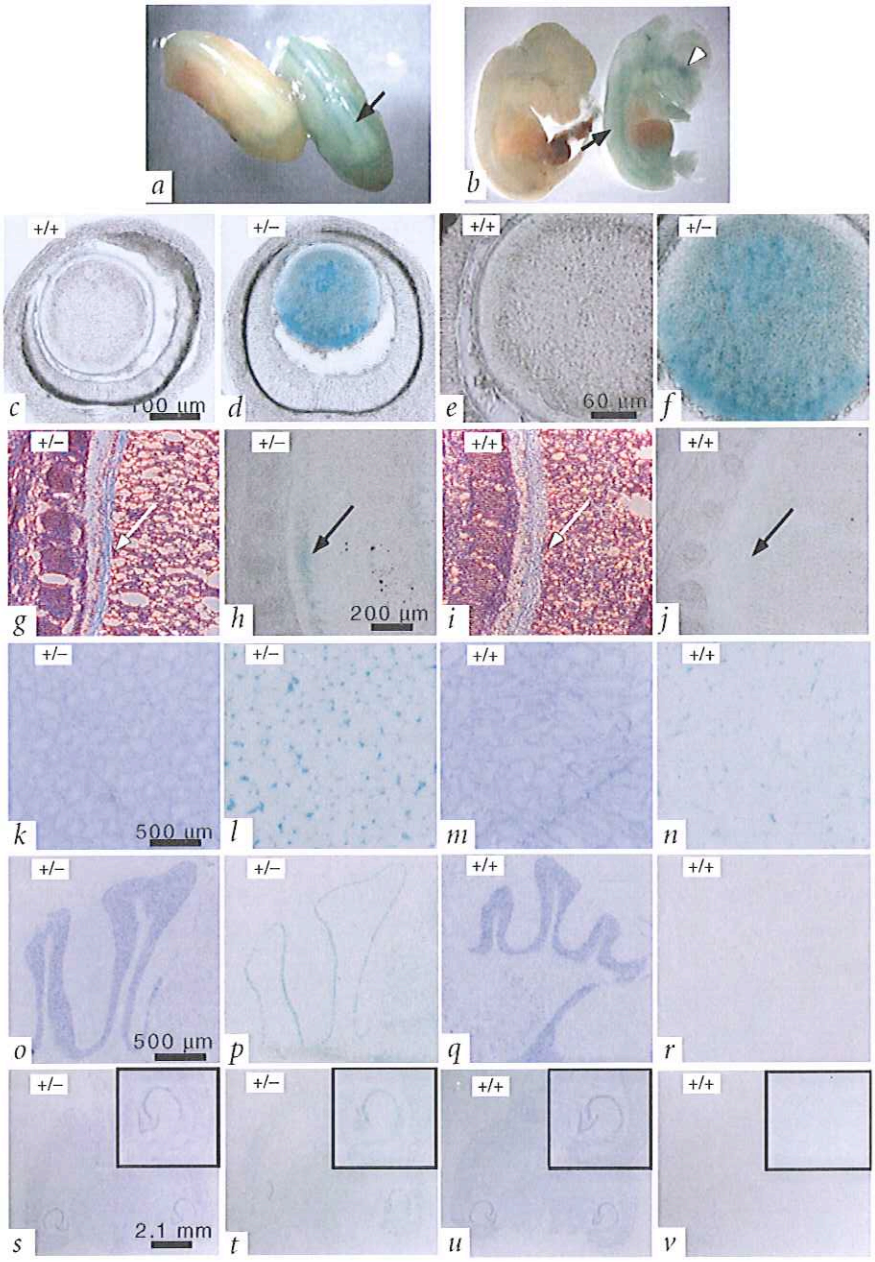


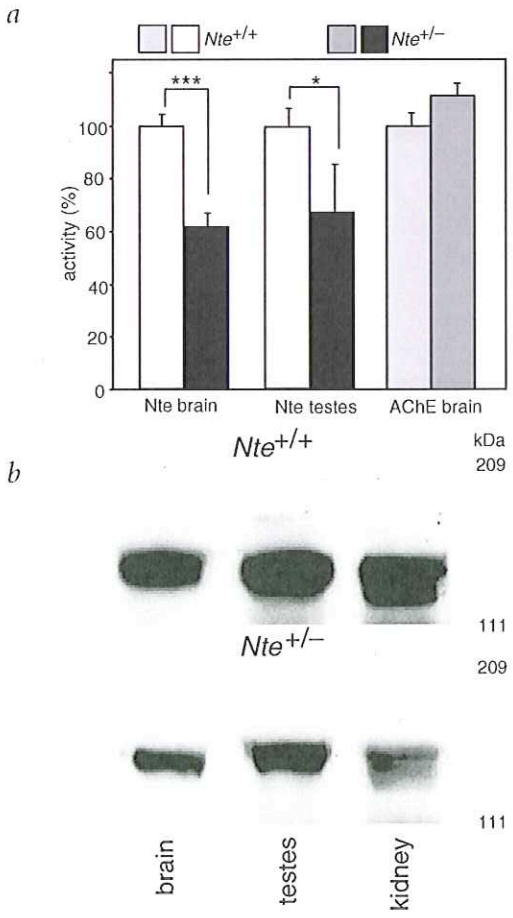
Fig. 4 *Nte* is highly expressed in developing spinal cord and lens and in specific regions of the adult brain and testes. *a*,*b*, Whole-mount and sagittal sections of E13.5 embryos after β-galactosidase staining showed expression of *Nte* in the developing spinal cord (black arrow) and eye (white arrowhead). A wild-type embryo is shown on the left side of each panel and an *Nte*$^{+/-}$ embryo is on the right side of each panel. *c–f*, Thin sections of eye (40 µm) confirmed that high levels of *Nte* expression were present in the developing lens. Sections of eyes from an *Nte*$^{+/-}$ embryo (*d*,*f*) and a wild-type embryo (*c*,*e*) were stained with X-gal. *g–j*, Sections (40 µm) of E13.5 embryos stained by Nissl (*g*,*i*) or X-gal (*h*,*j*), highlighting *Nte* expression in the developing spinal cord (arrows). *k–n*, *Nte* was abundantly expressed in the Leydig cells, as shown by X-gal staining of testes from an *Nte*$^{+/-}$ mouse (*l*). Low levels of non-specific X-gal staining were visible in testes from a wild-type mouse (*n*). Nissl staining of testes is shown in *k* and *m*. *o–r*, *Nte* was highly expressed in the Purkinje cell layer of the cerebellum as shown by X-gal staining (*p*,*r*). Cerebellar morphology can be observed in adjacent Nissl-stained sections (*o*,*q*). *s–v*, Horizontal sections showed that *Nte* seemed to be neuronal and was expressed in restricted regions throughout the brain, as indicated by X-gal staining in brains from *Nte*$^{+/-}$ mice (*t*) and wild-type mice (*v*). In particular, expression was observed in the cortex and CA1–CA3 of hippocampus but not in the striatum or dentate gyrus (insets). Nissl sections show neuronal morphology (*s*,*u*).

monitored clinical signs of mice treated with organophosphates and assessed mortality by Kaplan–Meier plot. The $Nte^{+/-}$ mice had significantly higher mortality rates at both 6 and 10 mg EOPF per kg body weight (Fig. 6*a*,*b*). Responses to EOPF included tearing, diminished movement and seizures. For comparison, no mortality or neuropathological effects were observed in either wild-type or $Nte^{+/-}$ mice treated with 1 mg EOPF per kg body weight. These results indicate that EOPF acts through direct inhibition of Nte activity and that the loss of Nte function leads to EOPF-induced toxicity.

Moderate reduction of Nte activity leads to greater motor activity

We measured several aspects of neurological function in wild-type and $Nte^{+/-}$ mice in the presence and absence of a low dose of EOPF (1 mg per kg body weight). In baseline studies without EOPF there were no significant differences between $Nte^{+/-}$ and wild-type mice in learning and memory, assayed by passive avoidance and audible conditioning experiments or response to a novel environment in standard open-field testing (data not shown). Based on these short-term measurements, $Nte^{+/-}$ mice showed no gross deficiencies in learning and memory or anxiety. Because of possible subtle latent effects, however, we extended our studies to examine long-term behavioral differences. We measured the locomotor activity of wild-type and $Nte^{+/-}$ mice (with or without exposure to 1 mg EOPF per kg body weight) by open-field chamber testing after three days of acclimatization to the chambers. We recorded the total distance traveled and vertical counts over a ten-day interval. $Nte^{+/-}$ mice showed higher baseline levels of motor activity than did wild-type mice, in terms of both distance traveled and vertical rearing (Fig. 6*c*,*d*). This is consistent with a phenotype of hyperactivity.

Because it was unclear whether this was due to lower Nte activity or developmental effects, we exposed the mice to 1 mg EOPF per kg body weight and recorded their locomotor activity. After exposure to EOPF, the $Nte^{+/-}$ mice had markedly lower activity levels (Fig. 6*c*,*d*), probably owing to further reduction in their already-compromised Nte levels. The wild-type mice dosed with 1 mg EOPF per kg body weight had significantly greater locomotor activity in terms of both total distance traveled (Fig. 6*c*) and vertical rearing (Fig. 6*d*). The difference in activity was almost identical to that seen at baseline (before exposure to EOPF) in the $Nte^{+/-}$ mice. Histological examination of spinal cord and brain samples from wild-type and $Nte^{+/-}$ mice at baseline and after treatment with 1 mg EOPF per kg body weight did not show any marked differences (data not shown). Taken together, these findings indicate that even partial inhibition of NTE activity (either chemically or genetically) leads to an abnormal neurological phenotype in mammals.



Discussion

It has been proposed that long-term repeated exposure to NTE inhibitors can lead to permanent chronic neuropsychopathological disease affecting behavior as well as cognitive and visual functions (ref. 1; for review see ref. 26). It has been further postulated that neurotoxic chemical combinations, such as pesticides and an insect repellent, may cause more severe brain dysfunction[3,27]. Additional evidence suggests that pesticide exposure may lead to neurobehavioral effects such as attention-deficit hyperactivity disorder (refs. 28,29). Several important questions have yet to be answered in establishing the precise role of NTE and the consequences of its inhibition in mammals. Most importantly, the physiologic function of NTE is unknown. The generation of mice with *Nte* haploinsufficiency confirms that inhibition of Nte activity, either genetically or chemically, leads to neurological effects.

NTE maps to human chromosome 19p13.3. Two human disease loci have been reported in this region, although neither is associated with disruption of *NTE* (see Online Mendelian Inheritance in Man). The mutation responsible for Weill–Marchesani syndrome at 19p13.3–13.2 mapped to the *WMS* gene. The human mucolipidosis IV mutation at 19p13.3–13.2 mapped to the gene encoding mucolipin I.

We also identified a second member of the family, *NTE-R1*, on human chromosome 9 and mouse chromosome 2. The function of NTE-R1 is unknown, although it may be related to esterase activities in different fractions of hen brain and sciatic nerves[30–32]. Our results clearly show that Nte-R1 cannot substitute for Nte during embryonic development and that it is not responsible for the effects of EOPF. The predicted NTE-R1 protein has a catalytic domain sharing homology with the NEST/patatin domain of NTE, but NTE-R1 functional esterase activity and substrate interactions have not yet been defined.

Fig. 5 $Nte^{+/-}$ mice had lower activity of Nte but not acetylcholinesterase. *a*, $Nte^{+/-}$ mice had approximately 40% ($P < 0.0001$) less activity of Nte in brain (solid black bar ± s.e.m., $n = 22$) and 33% ($P < 0.05$) less in testes (solid black bar ± s.e.m., $n = 6$) compared with wild-type littermates (solid white bars ± s.e.m.). In contrast, activity of acetylcholinesterase (AChE) was not significantly different between $Nte^{+/-}$ mice (hatched black bar ± s.e.m.) and wild-type littermates (hatched white bar ± s.e.m., $n = 22$). *b*, Nte protein levels in brain, testes and kidney were lower in $Nte^{+/-}$ versus wild-type mice as analyzed by immunoprecipitation with a mouse Nte-specific antibody. A band predicted to be roughly 145 kDa in size was observed.

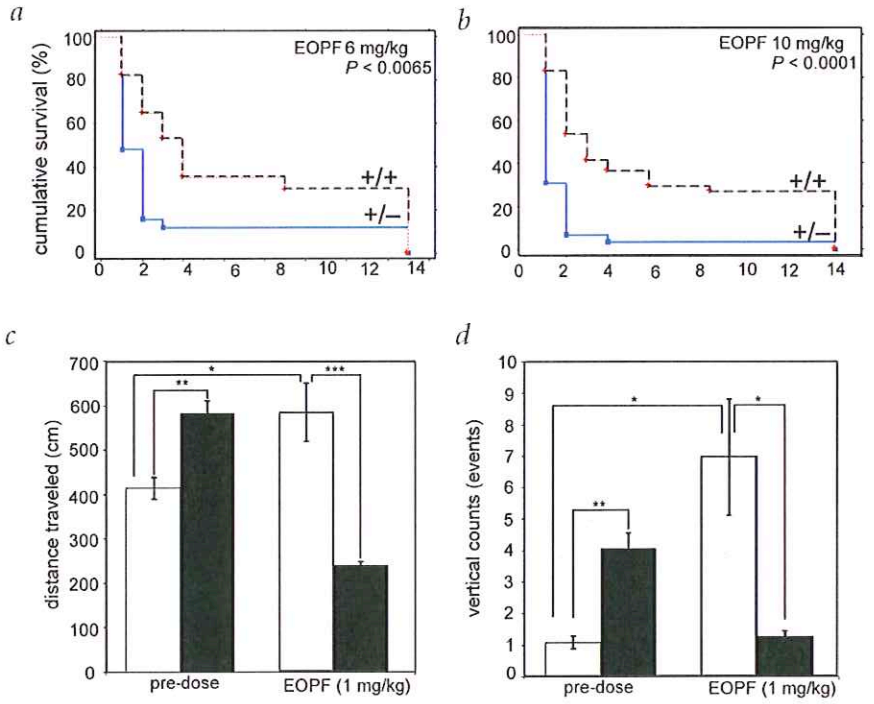


Fig. 6 *Nte*$^{+/-}$ mice were more sensitive to organophosphate toxicity and showed greater motor activity. *a,b*, Kaplan–Meier plots show that *Nte*$^{+/-}$ mice (solid line) had significantly higher mortality rates compared with wild-type littermates (dashed line) after exposure to EOPF. Significantly higher sensitivity was observed after treatment with 6 mg EOPF per kg body weight (mg/kg) for *Nte*$^{+/-}$ mice (n = 25) compared with wild-type mice (n = 17; $P < 0.0065$; *a*). Similar results were seen after treatment with 10 mg EOPF per kg body weight in *Nte*$^{+/-}$ mice (n = 29) compared with wild-type mice (n = 41; $P < 0.0001$; *b*). *c,d*, Motor activity in an open-field chamber as both distanced traveled (*c*) and vertical counts (*d*) was recorded at baseline and after challenging with EOPF (1 mg per kg body weight). The *x* axis represents genotype and EOPF exposure, and the *y* axis indicates motor activity. Baseline motor activity (before exposure to EOPF; pre-dose) was higher in *Nte*$^{+/-}$ mice (black bars ± s.e.m., n = 14) compared with wild-type mice (white bars ± s.e.m., n = 18). After treatment with EOPF, motor activity was higher in wild-type mice (white bars ± s.e.m., n = 4) and lower in *Nte*$^{+/-}$ mice (black bars ± s.e.m., n = 5). Significance was determined for all results presented using an unpaired *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Nte is highly expressed not only in hippocampal neurons, in the Purkinje cells of the cerebellum and in the spinal cord, but also in the Leydig cells of the testes and in the developing lens. Therefore, there probably are organismal effects of reducing NTE activity outside of the nervous system. Notably, the organophosphate phosphamidon leads to toxic effects in Leydig cells and clear cells of the cauda in the testes of rats[33], and environmental exposure to diazinon leads to retinal cell necrosis in teleosts[34]. Our results show that *Nte* is essential for embryonic survival, pointing to important consequences of inhibition of NTE in the developing vertebrate embryo and highlighting the key roles for NTE in mammalian embryogenesis and development.

A seminal observation is that moderate reduction in Nte activity, by either reducing the amount of Nte protein through genetics or using a potent Nte inhibitor, leads to hyperactivity. The mechanism of this possible cause-and-effect relationship has yet to be determined. The biochemical function of NTE is unknown, but considerable progress has been made recently in defining its physiological substrate. The catalytic domain of NTE alters membrane conductivity in liposome preparations, and neuropathic organophosphates change this response *in vitro*[35]. Human NTE hydrolyzes membrane associated lipids, pointing to a role for NTE in lipid metabolism or signaling[36]. NTE contains several cNMP-binding domains commonly found in intracellular signaling factors and cyclic nucleotide gated ion channels[10,36,37]. There is a good homology of the key residues around the active sites of NTE and calcium-independent phospholipase A2 (ref. 36). In this context, it is important to note that attention-deficit hyperactivity disorder has been linked to disorders involving lipid metabolism[38], pesticide exposure[28,29] and changes in ion-channel conductance in rat models[39]. Taken together, this evidence suggests that in mammals, the catalytic activity of NTE is linked to the control of motor activity and that the inhibition of NTE in humans may contribute to some hyperactivity disorders.

The neuropathies in hens arising from organophosphate inhibition of NTE cause wallerian axonal degeneration and show vacuolation and neuronal swelling over a period of 1–3 weeks[40,41]. Although the hen is the preferred model for OPIDN studies, there is growing evidence that this model may not properly estimate the risk of organophosphate exposure in humans and other mammals[42] and does not address short- and medium-term toxicities of organophosphates or non-neuropathy long-term sequelae. It is important that mice do not give a typical OPIDN response[21] and the mechanistic relationship between OPIDN in hens and delayed toxicity in mice has not yet been determined. In our study, no signs of neuropathy were observed in the *Nte*$^{+/-}$ mice that had 40% reduction in Nte activity, consistent with the 70–90% inhibition of Nte activity normally associated with organophosphate-induced delayed toxicity in wild-type mice[19,25] and OPIDN in hens[11–13]. But the *Nte*$^{+/-}$ mice were more sensitive to organophosphate-induced subacute toxicity, manifested by greater mortality, making it difficult to assess the long-term effects of exposure to higher levels of EOPF.

Neuropathology due to NTE inhibition by organophosphates is proposed to result from a toxic gain of function on aging of phosphorylated NTE and not merely from a loss of normal enzymatic activity[10,23]. Such a toxic gain of function is implicated in several neurodegenerative diseases, including familial amyotrophic lateral sclerosis[43], and in CAG triplet diseases, such as Huntington disease[44]. But our results show that even partial inhibition of Nte results in a clear neurobehavioral phenotype of hyperactivity. The experiments with Nte-deficient mice show that it is the reduction in Nte activity and not the generation of aged Nte that is responsible for the phenotype. The reduced level of Nte protein in the *Nte*$^{+/-}$ mice makes them more susceptible to the toxic effects of EOPF, establishing that it is a loss rather than a gain of function that results in toxicity. In addition, this study shows that chemical inhibition of Nte by EOPF mimics the phenotype caused by partial loss of Nte through genetic haploinsufficiency. Therefore, we clearly show for the first time that organophosphates that can cause neurological effects act through inhibition of Nte (without the requirement for aging or

gain of function) and this inhibition may be detrimental to the nervous system of mammals. The Nte-haploinsufficient mouse model will help define the relationship between NTE, delayed toxicity and OPIDN and may also be a useful model for understanding how nerve gas poisons and pesticides act individually or in combination to cause neurological dysfunction in man.

More than half a century of research with organophosphate toxicants has led to the discovery of several toxicological manifestations not attributable to acetylcholinesterase inhibition[1], including Gulf War syndrome[1,6,26,45]. NTE inhibition may be involved in some of these secondary events. The generation of mice lacking Nte is an important step in defining its function in mammals. The use of Nte-deficient mice may hasten the development of therapies for organophosphate-induced delayed toxicities and neuropathies. These findings show that *Nte* is highly expressed in the nervous system, that *Nte* is essential for embryonic development and survival and that mice lacking one allele of *Nte* are viable and fertile but have lower enzyme activity in the brain and are more susceptible to EOPF toxicity. Furthermore, a moderate reduction in Nte activity, either genetically or by administration of EOPF, is sufficient to produce hyperactivity. These observations provide biological evidence of the need to redefine the relationship between NTE, delayed toxicity and OPIDN in mammals. Clearly, *Nte* is an essential gene for development and for regulating motor activity.

Methods

Gene targeting and production of *Nte*-disrupted mice. We used a mouse EST to design PCR primers to screen a 129S6 mouse genomic BAC library (Incyte Genomics, Down-to-the-well) according to manufacturer's directions. PCR yielded a genomic fragment of 1122 bp and a cDNA fragment of 169 bp. Primer sequences are available on request. The BAC clone 59B13 was identified and characterized (Fig. 1*c*). We obtained and characterized the human BAC 48M5 (Incyte Genomics). We used a 160-bp probe to screen a liver cDNA library (Lifetech) and identified a 1659-bp alternatively spliced clone in addition to the full-length *Nte* (Fig. 1*c*; ref. 15). Isolation of a 5-kb *Bam*HI–*Bst*EII fragment containing exons 1–3 and a 5-kb *Sma*I–*Eco*RI fragment containing exons 10–15 enabled us to generate the targeting construct . We added a *Bam*HI linker to the *Bst*EII site in exon 3 and fused this in-frame to the *lacZ* gene. The *Nte–lacZ* fragment was inserted into the pPNT/PGK*Neo* vector[46] at an *Xho*I site, and the *Sma*I–*Eco*RI fragment was inserted as a blunt fragment into the *Kpn*I site of pPNT/PGK*Neo* adjacent to the thymidine kinase (*HSV-TK*) gene (Fig. 2*a*,*b*). We linearized and transfected the vector into 129S6 ES cells as previously described[47]. Using an internal probe and *Bst*EII digestion for Southern-blot analysis, we screened 114 G418-resistant and gancyclovir-sensitive ES cell clones (Fig. 2*a*). We identified nine positive clones. Extensive PCR, restriction-enzyme digestion and Southern-blot analyses with both internal and external primers and probes ensured selection of ES clones containing the correctly targeted locus (data not shown). We microinjected targeted clones into C57BL/6J blastocysts using standard methods. Two clones yielded chimeras capable of high-level germline transmission of the disrupted *Nte* allele as determined by mating with C57BL/6J mice. We established two inbred 129S6 lines harboring the disrupted *Nte* allele from the independent ES clones. We carried out genotyping by Southern-blot analysis as described above (Fig. 2*c*) and by PCR using a forward primer for a site 5′ to the targeted region and reverse primers for a site in the targeted region and in the *lacZ* gene (Fig. 2*d*). All animal procedures were carried out according to protocols approved by the Salk Institute for Biological Studies Animal Care and Use Committee.

FISH analysis. We prepared metaphase spreads for FISH on glass slides using standard protocols. We incubated cells in 0.1 mg ml^{-1} colcemid (GIBCO/BRL) for 30–60 min and then lysed them in 0.075 M KCl. We fixed chromosomes in 3:1 methanol:acetic acid and dropped them onto glass slides. We generated probes for FISH using BAC clones containing the genes of interest. We generated labeled BAC probes using the BioProbe nick-translation kit (Sigma). The BAC DNA clones were labeled with biotin-16-dUTP, digoxigenin-11-dUTP (Roche) or Spectrum Orange-dUTP (Vysis). Nick-translated probe DNA (100 ng) was precipitated with 15 μg mouse C_0t-1 DNA (Gibco) and resuspended in 50% formamide, 10% dextran sulfate, 2× saline-sodium citrate buffer. The probe DNA was denatured (10 min at 75 °C) and metaphase spreads were pretreated with RNase A (0.1 mg ml^{-1} for 1 h at 37 °C) and pepsin (0.1 mg ml^{-1} for 10 min at 37 °C) and then fixed in formalin (1% for 10 min at room temperature). After 30 min preannealing of probe DNA, we hybridized it to metaphase spreads for 24 h at 37 °C in a humidified box[48]. After hybridization, we detected indirectly labeled probes with either mouse antibody against digoxigenin followed by sheep antibody against mouse Cy5.5 or avidin–fluorescein isothiocyanate. FISH results were imaged and analyzed using QFISH software (Leica).

Staining for β-galactosidase activity. We obtained sections (10 μm) of fresh frozen tissue samples from wild-type and $Nte^{+/-}$ mice, fixed them in GTS fixation solution and visualized activity of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) using the X-gal Staining Assay Kit according to the manufacturer's directions (Gene Therapy Systems). We collected wild-type and $Nte^{+/-}$ embryos into phosphate-buffered saline at 4 °C. We fixed the embryos for 30 min in GTS fixation buffer and then incubated them three times for 10 min in a detergent rinse (0.1 mM phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% Nonidet P-40) before staining for X-gal activity as described above. We also prepared sections (40 μm) of embryos stained with X-gal to enable additional visualization of the developing lens and spinal cord. Nissl staining was carried out according to a standard protocol.

Activities of Nte and acetylcholinesterase. We homogenized brain (20% w/v) and testes (10% w/v) in 50 mM Tris buffer (pH 8.0) containing 0.2 mM ethylenediamine tetraacetic acid (EDTA). Homogenates were centrifuged at 700*g* for 10 min, pellets discarded and supernatants used for Nte and acetylcholinesterase assays. NTE is considered to be that portion of the phenyl valerate–hydrolyzing activity that is insensitive to paraoxon (40 μM) but sensitive to mipafox (50 μM). We determined activity of mouse brain Nte at 37 °C by a modification of the procedure for hen brain NTE[19,49]. Homogenates (50 μl) were diluted 20-fold in Tris-EDTA buffer (as above; 950 μl). Paraoxon (11 μg, 40 μM final concentration) was added in acetone (10 μl) and mipafox (0 or 9.1 μg, 50 μM final concentration) was added in acetone (10 μl). After 20 min incubation, phenyl valerate (480 μg) was introduced in 0.03% Triton X-100 in Tris-EDTA buffer (1 ml) and incubated for 15 min. The reaction was stopped with 1% SDS and 0.025% 4-aminoantipyrine in water (1 ml). Adding 0.4% potassium ferricyanide in water (0.5 ml) and waiting 5 min allowed colorimetric determination of Nte activity at 510 nm. A_{510} values for wild-type brain (n = 14) were 1.02 ± 0.08 with paraoxon and 0.68 ± 0.05 with paraoxon plus mipafox, giving Nte activity of 0.34 (about 10% of the total phenyl valerate–hydrolyzing activity). Testes had more variable Nte activity assays with A_{510} values of 0.92 ± 0.10, 0.78 ± 0.12 and 0.15, respectively (n = 13). We assayed acetylcholinesterase activity with acetylthiocholine (0.6 mM) and 5,5′-dithio-bis(2-nitrobenzoic acid) (1 mM) in 100 mM phosphate (pH 7.4, 1.1 ml) to which the undiluted homogenate (20 μl) was added and the absorbance increase continuously monitored over a period of 10 min at 412 nm[50].

Immunoprecipitation and determination of protein expression. We generated column-purified mouse Nte-specific antibodies against a peptide corresponding to amino acids 34–52 (RLRVQKTPAPEGPRYRFRK; Alpha Diagnostics). We homogenized tissue in Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, protease inhibitors (aprotinin + leupeptin), 1 mM phenylmethylsulfonyl fluoride) at 4 °C. The samples were then centrifuged and the supernatant was removed and stored. The protein supernatant (1 mg) was pre-cleared with Protein-A Sepharose slurry for 30 min, then removed and incubated with the Protein-A Sepharose for 2 h with the mouse Nte antibody (1:125 concentration). We removed the Protein-A Sepharose beads and washed samples, and protein was liberated on incubation with SDS buffer at 70 °C for 10 min. The supernatant was then run on a Tris-acetate gel, blotted and visualized with a secondary horseradish peroxidase–conjugate antibody and detected using the ECL kit (Amersham). Extra bands were observed in addition to the one corresponding to Nte, precluding use of this antibody for immunohistochemistry.

EOPF administration and toxicity studies. We dissolved EOPF (99% pure; synthesized in G.B.Q. and J.E.C.'s laboratory) in dimethylsulfoxide immediately before administration to the test mice. Mice were weighed immediately before EOPF treatment and were given a single intraperitoneal injection of EOPF corresponding to 1, 6 or 10 mg per kg body weight. Control mice received an injection of an equivalent volume of dimethylsulfoxide. We monitored the activity, appearance and mortality of the mice twice daily and measured weight daily during the course of the experiments.

Behavioral testing and assessment of locomotor activity. We carried out behavioral experiments measuring distance traveled and vertical counts using Open Field Test Chambers (Med Associates model ENV-515) of clear Plexiglas (43.2 cm × 43.2 cm × 30.5 cm) to monitor open-field activity in the light phase of the test mice's light/dark cycle. We took the mice from the home cage, placed them into the behavioral testing chamber for 15 min and then removed them directly back to the home cage. The open-field test chambers measured ambulatory distance, ambulatory counts, vertical counts and the time of each activity by monitoring light beams broken during the movement. We analyzed the data using Activity Monitor version 4.0 (Med Associates). We carried out standard open-field measures as described[46]. For total locomotor activity assays, we tested mice once per day over consecutive days. For baseline measurements, we tested 18 wild-type (9 female and 9 male) and 14 $Nte^{+/-}$ (6 female and 8 male) mice. We tested 4 wild-type (3 female and 1 male) and 5 $Nte^{+/-}$ (4 female and 1 male) mice that were treated with 1 mg EOPF per kg body weight. The testing trials lasted over a 12-d period. The first 2 d were for acclimation and thus were omitted from the total locomotor activity analysis. The data from the first 2 d were analyzed separately for behavioral differences at 5, 10 and 15 min intervals but showed no difference between the groups. We averaged the measurements of all mice of each group and activity over 10 d (after acclimation) to compare wild-type with $Nte^{+/-}$ mice.

We carried out passive avoidance tests for 2 d using a chamber (Med Associates, ENV-010MC) with two rooms, one brightly lit and one dark, separated by a small door. On day 1, we placed mice on the lighted side of the chamber for 10 s, opened the door to enable them to enter the dark side of the chamber, shut behind them and applied a foot shock for 3 s. We then removed the mice from the chamber and returned them to their home cage. On day 2, we again placed mice on the lighted side of the chamber and opened the door after a 10-s delay and then measured latency to enter. After the mice entered the dark side of the cage, the door was again shut but no shock was applied. We then returned the mice to their home cage. We applied foot shocks of 0.5 mA (wild-type, $n = 29$; $Nte^{+/-}$, $n = 31$) and 0.75 mA (wild-type, $n = 17$; $Nte^{+/-}$, $n = 13$) in separate experiments.

We carried out fear conditioning according to standard protocols. The test measures freezing responses in mice over a 7-d testing period. We used a shocking chamber (Med Associates, ENV-010MC) and a clear plexiglass chamber to test the mice. On day 1, we placed mice in the shocking chamber. After 160 s, three tone–shock pairs were delivered at intervals of 1 min. A tone–shock pair consists of 20 s of white noise followed by a 1-s 0.75-mA foot shock. After the tone–shocks, we kept the mice in the chamber for 60 s before returning them to the home cage. On day 2, we placed mice back in the shocking chamber 6 min and measured freezing to the context. On day 3, we put the mice into the clear plexiglass chamber. After 160 s, we delivered three 20-s tones identical to those in the tone–shock pairs delivered on day 1. The trial lasted for a total of 6 min. Freezing before the tones was considered a response to an unconditioned context. Freezing after the tones were delivered was considered a response to the tone. We conducted context extinction trials on days 4–7. These trials consisted of placing the mice back into the shocking chamber for 6 min each day. Freezing, which was measured as complete lack of movement in the body and minimal movement of the head, was timed using a stopwatch. We tested wild-type ($n = 4$) and $Nte^{+/-}$ ($n = 4$) mice.

Statistical analysis. We generated Kaplan–Meier survival plots to analyze the mortality data and measured significance using the Breslow–Gehan–Wilcoxon test, the Tarone–Ware test, the Peto–Peto–Wilcoxon test and the Harrington–Fleming test (rho = 0.5). Significant differences in Nte and acetylcholinesterase activities, behavioral distance traveled and vertical counts were determined using an unpaired *t*-test. One asterisk represents $P < 0.05$; two asterisks, $P < 0.01$; and three asterisks, $P < 0.001$.

Sequence and bioinformatics information. Analyses of the Celera Discovery System and NCBI databases were in agreement with our cytogenetic and sequence data. We searched the NCBI public databases (including LocusLink, GenBank, Online Mendelian Inheritance in Man, Mouse-Human Homology Maps and Unigene) and the Mouse Genome Database. In addition, we generated data using the Celera Discovery System and Celera's associated databases.

URLs. NCBI, http://www.ncbi.nlm.nih.gov; Mouse Genome Database, http://www.informatics.jax.org.

Accession numbers. The accession numbers for nucleic acid sequences described in this study are as follows: mouse *Nte* 5′ EST, NCBI W18687; the *NTE-R1* locus from mouse, NCBI NM_146251 (partial) and Celera mCG20285; rat, NCBI NM_144738 (partial); and human, NCBI NM_152286 (partial) and Celera hCG1811431.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Evidence that mouse brain neuropathy target esterase is a lysophospholipase

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Neuropathy target esterase (NTE) is inhibited by several organophosphorus (OP) pesticides, chemical warfare agents, lubricants, and plasticizers, leading to OP-induced delayed neuropathy in people (>30,000 cases of human paralysis) and hens (the best animal model for this demyelinating disease). The active site region of NTE as a recombinant protein preferentially hydrolyzes lysolecithin, suggesting that this enzyme may be a type of lysophospholipase (LysoPLA) with lysolecithin as its physiological substrate. This hypothesis is tested here in mouse brain by replacing the phenyl valerate substrate of the standard NTE assay with lysolecithin for an "NTE-LysoPLA" assay with four important findings. First, NTE-LysoPLA activity, as the NTE activity, is 41–45% lower in *Nte*-haploinsufficient transgenic mice than in their wild-type littermates. Second, the potency of six delayed neurotoxicants or toxicants as *in vitro* inhibitors varies from IC_{50} 0.02 to 13,000 nM and is essentially the same for NTE-LysoPLA and NTE ($r^2 = 0.98$). Third, the same six delayed toxicants administered i.p. to mice at multiple doses inhibit brain NTE-LysoPLA and NTE to the same extent ($r^2 = 0.90$). Finally, their *in vivo* inhibition of brain NTE-LysoPLA generally correlates with delayed toxicity. Therefore, OP-induced delayed toxicity in mice, and possibly the hyperactivity associated with NTE deficiency, may be due to NTE-LysoPLA inhibition, leading to localized accumulation of lysolecithin, a known demyelinating agent and receptor-mediated signal transducer. This mouse model has some features in common with OP-induced delayed neuropathy in hens and people but differs in the neuropathological signs and apparently the requirement for NTE aging.

Organophosphorus (OP) esters are the principal class of insecticides (1) and chemical warfare agents (2). Their acute lethal action is attributed to inhibition of acetylcholinesterase by phosphorylation of its catalytic site (3). The second most important toxic effect of these compounds and some related pesticides, lubricants, and plasticizers is OP-induced delayed neuropathy (OPIDN) (4–7). The principal causal agent for the >30,000 cases of human paralysis is tri-*o*-tolyl phosphate as an adulterant in beverages around 1930 and in cooking oil in 1959 (5–7), but concern continues because it is still a common component in commercial jet oils (8). The delayed neurotoxicity of this triaryl phosphate involves bioactivation to the *o*-tolyloxy derivative of benzodioxaphosphorin oxide (BDPO; ref. 9). Other OP delayed neurotoxicants are the insecticide mipafox, which is no longer used (4, 5), and the cotton defoliant tribufos (10). Their structures are given in Fig. 1.

The target for OPIDN is a nerve protein with esteratic activity that is phosphorylated and inhibited by the delayed neurotoxicant (4). Neuropathy target esterase (NTE) is present in human, hen, and mouse brain and is defined as the paraoxon-resistant and mipafox-sensitive esterase with phenyl valerate-hydrolyzing activity (4–6, 11–14). NTE inhibitors of varying potency are *o*-tolyloxy-BDPO, mipafox, and tribufos (indicated above) and ethyl octylphosphonofluoridate (EOPF; refs. 13 and 14), the octyl-BDPO enantiomers (14, 15), and dodecanesulfonyl fluoride (DSF; see ref. 16 for the octane analog) designed for potency (Fig. 1). An aging reaction also is required for OPIDN; e.g., *O*,*O*-diisopropylphosphoryl-NTE is O-dealkylated to *O*-isopropylphosphoryl-NTE (see *Discussion*). The NTE assay is fully validated for toxicological relevance to OPIDN (11, 12) and served as the monitoring procedure in NTE isolation (17, 18). When the esterase domain of NTE (residues 727–1216) is expressed as the recombinant polypeptide (designated as NEST; ref. 19), the preferred substrate is 1-palmitoyl-*sn*-glycero-3-phosphocholine (also known as 1-palmitoyllysophosphatidylcholine, a lysolecithin) with $K_m \approx 50$ μM (20). NEST has sequence similarity in the active site region to calcium-independent phospholipase A_2 (20), known to have lysophospholipase (LysoPLA) activity (21). Lysolecithin therefore becomes a candidate for the physiological substrate (ref. 20; Fig. 2).

Biochemical studies indicated above suggest that NTE may be a LysoPLA acting on lysolecithin. This hypothesis is tested here in mice by replacing the phenyl valerate substrate of the standard NTE assay with lysolecithin for a different type of "NTE-LysoPLA" assay (Fig. 2). Mouse brain was selected because OP-induced delayed toxicity in mice correlates with NTE inhibition (14, 16) and *Nte*-haploinsufficient ($Nte^{+/-}$) mice are hyperactive and more sensitive to the delayed toxicant EOPF (22). As with NTE, the paraoxon-resistant and mipafox-sensitive LysoPLA activity was considered most important. A simple and specific assay was developed for NTE-LysoPLA. Brain preparations from wild-type and $Nte^{+/-}$ mice first were examined for activity with lysolecithin as the substrate. Then a series of OP toxicants of diverse structures (Fig. 1) and potencies was examined. NTE-LysoPLA and NTE should be similarly inhibited by these compounds *in vitro* and *in vivo* if they are in fact the same enzyme. The dose–response effects, as *in vivo* enzyme inhibitors, should also correlate with delayed toxicity. These are severe tests for the possible assignment of mouse brain NTE as a LysoPLA.

Materials and Methods

Materials. Lysolecithin (primarily palmitate and stearate esters, derived from egg yolk), *sn*-glycero-3-phosphocholine phosphodiesterase (mold), choline oxidase (*Alcaligenes* sp.), peroxidase (horseradish), 3-(*N*-ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid sodium salt, and 4-aminoantipyrine were obtained from Sigma. Paraoxon, mipafox, and six delayed toxicants or neurotoxicants (Fig. 1) were available from previous studies in this laboratory.

Mice and Brain Enzyme Preparations. Male Swiss–Webster mice (≈25 g) were from Harlan Laboratories (Indianapolis). $Nte^{+/-}$ and the corresponding wild-type (+/+) mice (129S6/SvEvTac, male, ≈4 months old) were from the Salk Institute (22). The brain (freshly obtained or from storage at −80°C) was homog-

Abbreviations: OP, organophosphorus; OPIDN, OP-induced delayed neuropathy; BDPO, benzodioxaphosphorin oxide; NTE, neuropathy target esterase assayed with phenyl valerate as the substrate; EOPF, ethyl octylphosphonofluoridate; DSF, dodecanesulfonyl fluoride; LysoPLA, lysophospholipase; NTE-LysoPLA, NTE assayed with lysolecithin as the substrate; AU, absorbance units.

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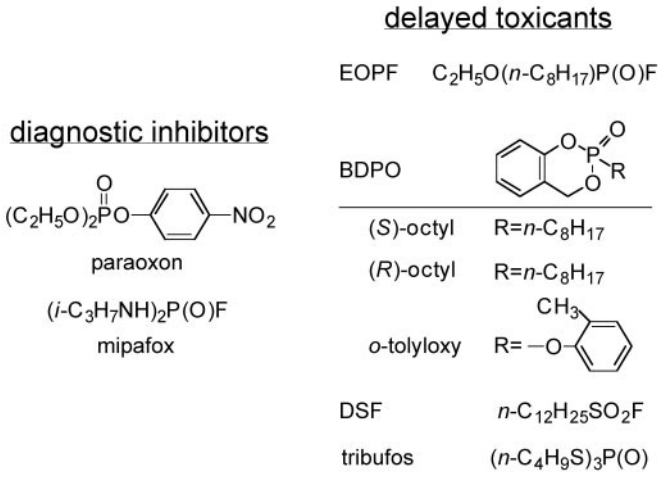

Fig. 1. Structures of OP diagnostic inhibitors and delayed toxicants or neurotoxicants.

enized at 20% (wt/vol) in 50 mM Tris buffer (pH 8.0) containing 0.2 mM EDTA at 4°C. Enzyme assays used the supernatant fraction (700 × *g*, 10 min) at 4°C within 6 h after preparation.

Enzyme Activity and Inhibition Assays. The substrate used for NTE-LysoPLA was lysolecithin and that for NTE was phenyl valerate. In both assays only the paraoxon-resistant and mipafox-sensitive portion of the activity is relevant. NTE-LysoPLA was assayed at 25°C, and NTE was assayed at 37°C, in each case after a 20-min pretreatment with paraoxon at 40 μM and mipafox at 0 or 50 μM. For IC_{50} determinations, inhibitors were added in dimethyl sulfoxide (5 μl) with a 15-min incubation before the introduction of paraoxon/mipafox. Unless specified otherwise, the data are averages of four to eight replicates (NTE-LysoPLA) or one to two determinations (NTE), reflecting the greater variability of the former method.

NTE-LysoPLA Activity Assays. LysoPLAs are OP-sensitive enzymes that hydrolyze lysolecithin to *sn*-glycero-3-phosphocholine (Fig. 2; refs. 23 and 24). The assay procedure is modified from a method for analysis of lysolecithin in human serum and plasma (25). LysoPLA activity is monitored continuously in an enzyme-coupled microplate assay in which only the first step is OP-sensitive. The reaction proceeds to form choline, hydrogen peroxide, and a colored derivative from the sequential action of three added enzymes (*sn*-glycero-3-phosphocholine phosphodiesterase, choline oxidase, and peroxidase) and two chromogenic agents. The enzyme assayed is either a LysoPLA or a phospholipase functioning as a LysoPLA.

NTE-LysoPLA activity is proportional to the paraoxon-resistant and mipafox-sensitive increase in absorbance at 570 nm. More specifically, unless indicated otherwise, all reactants were added in 100 mM Tris buffer (pH 8.0) containing 1 mM calcium chloride and 0.01% Triton X-100. Reagent A contains 3-(*N*-ethyl-3-methylanilino)-2-hydroxysulfonate (3 mM), peroxidase (10 units/ml), *sn*-glycero-3-phosphocholine phosphodiesterase (0.0001 units/ml), and choline oxidase (10 units/ml). Reagent B contains 5 mM 4-aminoantipyrine. Reagents A (120 μl) and B (80 μl) were added to individual chambers containing Tris buffer as above (45 μl) in a 96-well polystyrene plate. Brain homogenate (15 μl) was added, followed by paraoxon in acetone (5 μl) and mipafox in 50 mM Tris–citrate (5 μl) to give 40 and 50 μM final concentrations, respectively. After a 20-min incubation, lysolecithin (250 μM final concentration) is introduced in Tris buffer (50 μl). The NTE-LysoPLA activity is measured by kinetic assay of absorbance at 570 nm for 10 min at 25°C by using a microplate reader (Molecular Devices). The activity was linear with regard to protein level and time.

NTE Activity Assays. The procedure is a modification for mouse brain (14) of a standard method for hen brain (12). Brain homogenate (50 μl) was added to Tris–EDTA buffer (as above; 950 μl). Paraoxon and mipafox were introduced as before but in 10 μl of solution to give 40 and 50 μM final concentrations, respectively. After a 20-min incubation, phenyl valerate (1.4 mM final concentration) was introduced in 0.03% Triton X-100 in Tris–EDTA buffer (1 ml) and incubated for 15 min. The reaction was stopped with 1% SDS and 0.025% 4-aminoantipyrine in water (1 ml). Addition of 0.04% potassium ferricyanide in water (0.5 ml) allowed colorimetric determination at 490 nm of phenol liberated by NTE.

***In Vivo* Inhibition of NTE-LysoPLA and NTE Activities and Poisoning Signs.** Swiss–Webster mice were treated i.p. with the test compound in dimethyl sulfoxide (30–100 μl) or the carrier solvent alone as a control. They were killed after 4 h, and the brains were assayed for NTE-LysoPLA and NTE activities on the same preparations. Other mice in the treated group were rated for delayed toxic or neurotoxic signs (14) or death at 0.3–6 days (16).

Results

Diagnostic Inhibitors for Brain NTE-LysoPLA and NTE. Mouse brain NTE-LysoPLA and NTE are both insensitive to paraoxon (no inhibition at 10^5 nM) and moderately sensitive to mipafox (IC_{50} 5,700–13,000 nM; Fig. 3). These OP compounds therefore are used as diagnostic inhibitors, determining the paraoxon (40 μM)-resistant and mipafox (50 μM)-sensitive portion of lyso-

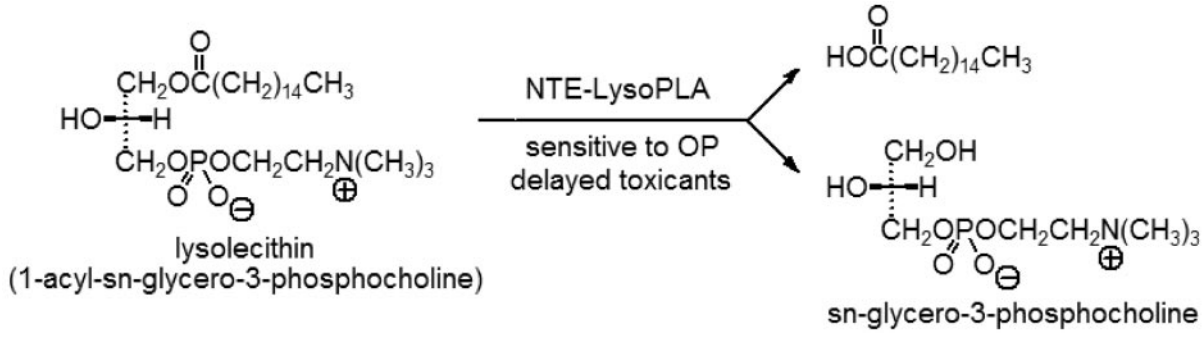

Fig. 2. Proposed biochemical lesion induced by OP delayed toxicants or neurotoxicants involving inhibition of NTE-LysoPLA, which normally hydrolyzes lysolecithin as its physiological substrate. The classical substrate for NTE is phenyl valerate, based on ease of assay (4, 12) rather than mechanistic considerations. NTE-LysoPLA and NTE in brain are resistant to paraoxon but sensitive to mipafox and other delayed toxicants or neurotoxicants.

A

compound	IC_{50} (nM) mouse NTE-LysoPLA	mouse NTE	hen NTE
diagnostic inhibitors			
paraoxon	$>10^5$	$>10^5$	$>10^5$
mipafox	13,000	5,700	7,000
delayed toxicants			
EOPF	0.05	0.02	0.04
(*S*)-octyl-BDPO	0.6	0.8 (0.05)	0.09
(*R*)-octyl-BDPO	2.0	2.3	7.8
o-tolyloxy-BDPO	80	150	60 (130)
DSF	470	250	

B

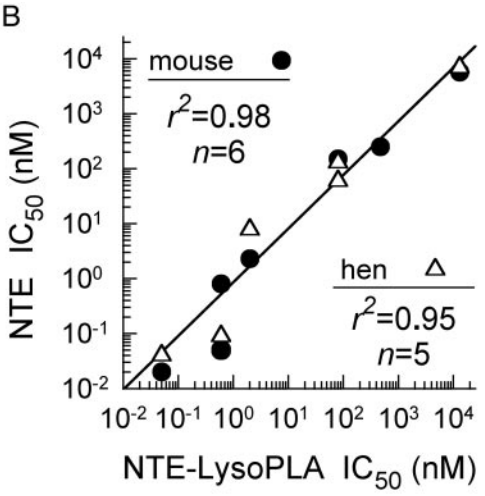

Fig. 3. Relationship between *in vitro* sensitivities of brain NTE-LysoPLA and NTE activities. NTE-LysoPLA and NTE were assayed with lysolecithin and phenyl valerate, respectively. Compound structures are shown in Fig. 1. (*A*) The data for mouse NTE-LysoPLA for all compounds and mouse NTE for paraoxon, (*S*)-octyl-BDPO, and DSF are from this study with Swiss–Webster mice. Other data for mouse NTE are from this laboratory (14, 16) or Veronesi *et al.* (26) for *o*-tolyloxy-BDPO. The hen NTE data are from Wu and Casida (13, 15, 27), Johnson (11), and Veronesi *et al.* (26). (*B*) Correlation for sensitivity of NTE-LysoPLA from mouse brain versus NTE from mouse brain (●) and hen brain (△). Correlation coefficients (r^2) do not include tabulated values for paraoxon ($>10^5$ nM) or those in parentheses, which were determined by a different investigator (0.05 nM for mouse NTE) or a different laboratory (130 nM for hen NTE).

lecithin and phenyl valerate hydrolysis, respectively. The LysoPLA activity values [milliabsorbance units (mAU)/min, $n = 13$, mean ± SD] for brain from Swiss–Webster mice are 18.4 ± 3.2 with paraoxon and 15.3 ± 1.9 with paraoxon plus mipafox, giving NTE-LysoPLA activity of 3.1 (17% of the total lysolecithin-hydrolyzing activity). The NTE activity values (AU, $n = 15$, mean ± SD) for Swiss–Webster mice are 0.60 ± 0.09 with paraoxon and 0.44 ± 0.06 with paraoxon plus mipafox, giving an NTE activity of 0.16 [10% of the total activity with no inhibitors, compared with 12% in our earlier report (14)]. These conditions therefore were standardized in assaying the mouse brain NTE-LysoPLA and NTE activities.

Relationship Between Brain NTE-LysoPLA and NTE Activities of NTE-Deficient Mice. Brain NTE-LysoPLA and NTE activities are somewhat higher for the wild-type 129S6/SvEvTac mice than for the Swiss–Webster mice given above. The LysoPLA activity values (mAU/min, $n = 7$, mean ± SD) for the +/+ mice are 14.3 ± 1.9 with paraoxon and 10.2 ± 1.4 with paraoxon plus mipafox, giving NTE-LysoPLA activity of 4.1 (≈29% of the total lysolecithin hydrolysis). The NTE activity values (AU, $n = 22$, mean ± SD) for the +/+ mice are 0.85 ± 0.07 with paraoxon and 0.53 ± 0.04 with paraoxon plus mipafox, giving an NTE activity of 0.32 (≈38% of the total phenyl valerate hydrolysis).

The brains of $Nte^{+/-}$ mice have 59% of the NTE-LysoPLA and 55% of the NTE activities of their wild-type littermates, i.e., highly significant reductions ($P < 0.01$) in both cases (Table 1). The similar reduction in activity for both NTE-LysoPLA and NTE with a single gene deletion indicates that the same enzyme is involved.

Relationship Between *in Vitro* Sensitivities of Brain NTE-LysoPLA and NTE Activities. Inhibitor specificity profiles provide a powerful method for differentiating one enzyme from another or establishing their identity. Six delayed toxicants or neurotoxicants, including mipafox, were examined along with the inactive paraoxon (Fig. 3*A*). Two of them [EOPF and (*S*)-octyl-BDPO] are highly potent OP inhibitors in both assays (IC_{50} 0.02–0.8 nM). The same enantiomeric specificity of octyl-BDPO is evident with both NTE-LysoPLA and NTE; i.e., the *S* enantiomer is ≈3-fold more potent than the *R* enantiomer when determined with mouse brain in the same experiment. *o*-Tolyloxy-BDPO is of intermediate potency, and DSF is less potent but still similar in both assays. The data are conveniently compared as a correlation plot for NTE-LysoPLA versus NTE of mouse brain ($r^2 = 0.98$, $n = 6$) or NTE-LysoPLA of mouse brain versus NTE of hen brain ($r^2 = 0.95$, $n = 5$) (Fig. 3*B*), providing evidence that NTE-LysoPLA is identical to NTE under the assay conditions.

Relationship Between *in Vivo* Inhibition of Brain NTE-LysoPLA and NTE Activities. Six delayed toxicants or neurotoxicants were administered to mice, and the inhibition of NTE-LysoPLA activity was compared with that of NTE activity 4 h later (Table 2). This comparison included EOPF, three BDPO derivatives, and DSF, which act directly, and tribufos, which undergoes bioactivation (28) and therefore was not included in the *in vitro* assays above. The six compounds, three at multiple doses, inhibited NTE-LysoPLA and NTE activities to the same extent ($r^2 = 0.90$, $n = 13$). Importantly, the high potency of EOPF and the same stereospecificity for (*R*)- and (*S*)-octyl-BDPO were observed for both assays, providing further evidence that NTE-LysoPLA and NTE are very similar or identical.

Relationship Between *in Vivo* NTE-LysoPLA and NTE Inhibition and Delayed Toxicity. Swiss–Webster mice in 12 groups were examined for a possible relationship between NTE-LysoPLA and

Table 1. Relationship between brain NTE-LysoPLA and NTE activities of NTE-deficient mice

Genotype*	NTE-LysoPLA, mAU/min†	NTE, AU†
Absolute activity		
+/+	4.09 ± 0.22	0.321 ± 0.018
+/−	2.43 ± 0.19‡	0.176 ± 0.045‡
Relative activity, %		
+/− ÷ +/+	59	55

*Nte heterozygous 129S6/SvEvTac ($Nte^{+/-}$) transgenic mice and their wild-type littermates.

†NTE-LysoPLA and NTE assayed with lysolecithin and phenyl valerate, respectively. $n = 7$ for +/+ and 4 for +/− in each case as the average of four assays for NTE-LysoPLA and two for NTE. Data are mean ± SE.

‡Significant difference ($P < 0.01$) for both NTE-LysoPLA and NTE (comparison of +/+ with +/−).

PHARMACOLOGY

Table 2. Relationship between *in vivo* inhibition of brain NTE-LysoPLA and NTE activities and delayed toxicity

Toxicant and dose, mg/kg	Enzyme inhibition, %*		Delayed toxicity
	NTE-LysoPLA	NTE	
EOPF			
1	18 ± 15	0 ± 0	−†
2	89 ± 12	89 ± 2	
3	100 ± 0	78 ± 5	+†
10	99 ± 1	95 ± 4	+†
(*S*)-octyl-BDPO			
5	71 ± 8	92 ± 7	+†
(*R*)-octyl-BDPO			
5	7 ± 8	6 ± 7	−†
o-Tolyloxy-BDPO			
3	20 ± 17	8 ± 4	−
10	70 ± 4	55 ± 13	−
30	89 ± 7	94 ± 7	−‡
100	87 ± 16	100 ± 0	−‡
DSF			
100	92 ± 9	100 ± 0	+
Tribufos			
30	7 ± 8	11 ± 9	−§
100	85 ± 16	100 ± 0	+§

Compounds were administered i.p. to Swiss–Webster mice with determinations of enzyme activities at 4 h and delayed toxicity at 3 days.

*NTE-LysoPLA and NTE were assayed with lysolecithin and phenyl valerate, respectively. $n = 3$ in each case as the average of duplicate assays for NTE-LysoPLA and single determinations for NTE. Data are mean ± SE.

†Ref. 14.

‡Cholinergic poisoning signs at 100, but not 30, mg/kg.

§Ref. 16.

NTE inhibition and delayed toxicity (Table 2). Five sets of treatments gave 71–100% NTE-LysoPLA inhibition and 78–100% NTE inhibition with delayed toxicity. Another five sets gave enzyme inhibition of 7–70% for NTE-LysoPLA and 0–55% for NTE without delayed toxicity. An apparent exception is *o*-tolyloxy-BDPO at 30 and 100 mg/kg, with 87–100% NTE-LysoPLA and NTE inhibition and cholinergic signs at the higher dose. Importantly, EOPF and (*S*)-octyl-BDPO were the most effective NTE-LysoPLA and NTE inhibitors both *in vitro* and *in vivo* (Table 2; Fig. 3), and they are also the most potent delayed toxicants. Thus, *in vivo* inhibition of NTE-LysoPLA and NTE activities is generally correlated with delayed toxicity.

Discussion

Assignment of Mouse Brain NTE as NTE-LysoPLA. Four lines of evidence support the assignment of mouse brain NTE as a type of LysoPLA, designated here as NTE-LysoPLA: (*i*) $Nte^{+/-}$ transgenic mice are similarly deficient in NTE-LysoPLA activity, (*ii*) the potency of delayed toxicants or neurotoxicants as *in vitro* inhibitors is essentially the same for NTE-LysoPLA and NTE, (*iii*) these compounds inhibit NTE-LysoPLA and NTE activities *in vivo* to the same extent, and (*iv*) toxicant-induced *in vivo* inhibition of NTE-LysoPLA is generally predictive of delayed toxicity. Based on the same OP pharmacological profile as NTE *in vitro* and *in vivo*, NTE-LysoPLA seems to play a key role in OP-induced hyperactivity and delayed toxicity in mice.

LysoPLAs: A Large Family of OP-Sensitive Enzymes. The human proteome contains >100 lipid hydrolases (29). Within this, the LysoPLAs are a large family of enzymes (21). Characterized isoforms are either small (≈25 kDa) or large (>50 kDa), and several have been cloned from mouse, rat, human, and rabbit sources (21). They are present in many tissues, and more than one isoform can exist in a single cell (23). The relative roles these and other LysoPLAs play in regulating lysolecithin and other lysophospholipid levels in cells are largely unknown (21). LysoPLAs are the principal enzymes for removing lysophospholipids from cell membranes, including in human brain (30). LysoPLAs act on substrates within nerve membranes (21), and the activity of NTE requires a membrane environment (19). NTE-LysoPLA represents only a small portion of the total LysoPLA activity. It has an apparent molecular mass, based on NTE, of ≈155 kDa (31, 32), which differs from previously identified LysoPLAs (21). LysoPLAs of ≈25 kDa are inhibited by OPs such as diisopropyl fluorophosphate for enzyme from mouse macrophage-like cells (IC_{50} 5 mM; ref. 23) and methyl arachidonyl fluorophosphonate (IC_{50} 600 nM) for human brain recombinant LysoPLA (24). Much higher sensitivity is observed in the present study to octylphosphonates EOPF and (*S*)-octyl-BDPO ($IC_{50} < 1$ nM) for NTE-LysoPLA from mouse brain. NTE-LysoPLA hydrolyzes lysolecithin (21) and thus determines its localization and persistence. The localization in nerve of NTE-LysoPLA is probably the same as that of NTE as defined by immunohistochemical techniques for mouse (22) and hen (33), histochemical demonstration of NTE esterase activity (34), and autoradiographic detection of [^{3}H]octyl-BDPO-labeled NTE for hen (35).

Lysolecithin, Receptor-Mediated Signal Transduction, and Demyelination. Lysophospholipids play an essential role in phospholipid metabolism, and *in vivo* levels are critical for cell survival and function (21). They are normally present at low concentrations in membranes (0.5–6% of total lipid weight), but under pathological conditions lysolecithin constitutes up to 40% of the total lipid, e.g., in atherogenic lipoproteins (21). Increased lysophospholipid levels are associated with a host of diseases such as hyperlipidemia, inflammation, and lethal dysrhythmias in myocardial ischemia (21). Lysolecithin is a major nerve constituent with several types of neuroactivity (23). It acts directly at G protein-coupled receptors and induces receptor-mediated signal transduction (36, 37). Most relevant here, lysolecithin causes demyelination of neuronal sheaths (also typical for human multiple sclerosis), often accompanied by axonal lesions as a rapid and localized effect (38–42). Interestingly, the OP delayed neurotoxicant diisopropyl fluorophosphate produces somewhat similar effects, inducing "chemical transection of the axon" and demyelination (5, 43), and direct treatment of one sciatic artery or nerve produces localized unilateral neuropathy (44, 45).

Relevance of Mouse Brain NTE-LysoPLA Model to OPIDN. Advances in determining NTE structure and function have come from studies with several species and levels of organization. Despite many common features, there appear to be species differences in the sequence of events between NTE inhibition and neurotoxicity. The *Drosophila* neurodegeneration gene *swiss cheese* encodes a neuronal protein involved in glial hyperwrapping and brain degeneration and homologous to human NTE, possibly relating it to OPIDN (46, 47). The mouse NTE protein is 96% identical to the human NTE protein (46). The primary neuropathological lesion of OPIDN in hens is a bilateral degenerative change in distal levels of axons and their terminals, mainly affecting larger/longer myelinated central and peripheral nerve fibers, leading to breakdown of neuritic segments and their myelin sheaths (5). Demyelination of nerve sheaths occurs in adult hens, whereas more restricted lesions are evident in rats (5). Mice respond somewhat differently than hens and humans to OP delayed neurotoxicants, with more rapid action (in 3–5 versus 10–14 days) and less pronounced neuropathy, i.e., delayed toxicity rather than OPIDN (5). However, mice have obvious advantages in mechanisms research (14, 22). The model considered here relates OP-induced hyperactivity (22) and delayed mortality (14) to inhibition of mouse brain NTE-LysoPLA

activity. This relationship in turn may result in a localized increase in lysolecithin level, which induces a cell signaling cascade, possibly leading to hyperactivity (or demyelination in sensitive species).

This mouse model has some features in common with OPIDN in hens and people but differs in the neuropathological signs and apparently the requirement for NTE aging. Unresolved questions (4–6) include the mechanism of species differences, not only in the time delay and neurotoxic/neuropathic signs, but also in the importance of dealkylation (aging) of the inhibited enzyme, possibly with a ''toxic gain of function'' in hens (4, 6, 48) but apparently not in mice (22), and the observation that some potent inhibitors are not neuropathic but protect against subsequent challenge with a neuropathic OP. The established relationships and the anomalies now can be viewed in a new light focusing on the inhibition of NTE-LysoPLA and localized elevation of lysolecithin as possible contributors to OP-induced delayed toxicity in mice or even to OPIDN in hens and people. Perhaps the LysoPLA activity of NTE is not the only OP-sensitive function of this 155-kDa protein.

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Neurotoxic esterase: not so toxic?

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An altered form of an esterase has been implicated in the development of neurotoxicity after exposure to organophosphates. Mice deficient in this enzyme should be less susceptible to toxicity, but the opposite turns out to be the case.

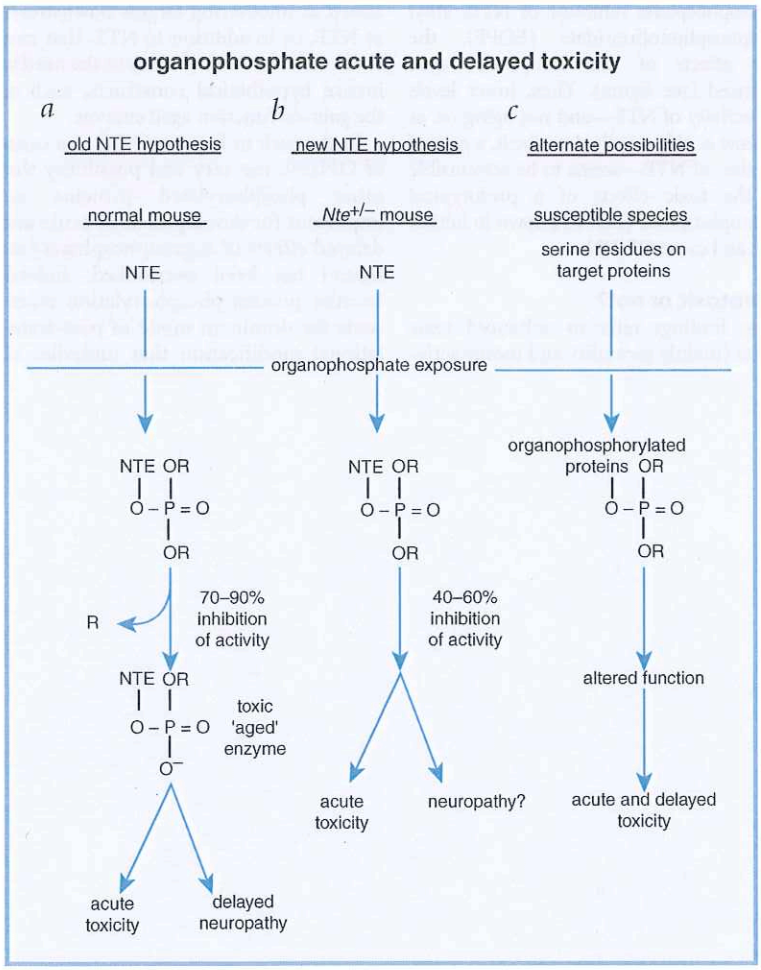
Although of recent concern because of their use as chemical warfare agents, organophosphates have long been used as pesticides. As a class of compounds, organophosphate esters are widely recognized for their potential to inhibit serine-containing esterases owing to phosphorylation of serine residues at the active site of these enzymes, the most notable of which is acetylcholinesterase. Most features of the acute toxicity of these compounds (used as nerve gas or as pesticides) relate to their inhibition of this enzyme.

A less well known feature of some organophosphates is their propensity to cause a delayed neuropathy that has been termed organophosphate-induced delayed neurotoxicity (OPIDN; ref. 1). OPIDN is a progressive neurological condition characterized by weakness, ataxia and subsequent paralysis of the limbs[1,2]. The major neuropathological hallmarks of OPIDN are degeneration of the long axons of the spinal cord and peripheral neurons, although assessments of neurotoxicity using sensitive stains for neurodegeneration show that selected regions of the brain are also involved[3].

For over 30 years, the proposed target for initiation of OPIDN has been an enzyme activity called neurotoxic esterase[4] or neuropathy target esterase (NTE; ref. 5). Despite decades of research, NTE has only recently been identified as a *bona fide* protein[6]. The mechanistic basis for initiation of OPIDN through NTE is largely operationally defined: the key feature of the NTE hypothesis of OPIDN involves the generation of an 'aged' form of the enzyme (ref. 5; see figure). In the accompanying paper, however, Christopher Winrow and colleagues[7] provide evidence against the aging concept without ruling out involvement of NTE itself.

Out with the old

So, just what is aged NTE and why is it required for OPIDN? The aged NTE concept was conceived when it was discovered that not all organophosphates that



Revisiting the NTE hypothesis. Pathways leading to acute toxicity and delayed neuropathy associated with exposure to organophosphates. According to a long-standing concept in neurotoxicology, the activity of a target esterase (neurotoxic esterase or neuropathy target esterase) is inhibited by organophosphorylation at a key serine residue (refs. 4,5; *a*). When inhibition of enzyme activity reaches 70–90% and, when phosphorylated NTE is modified by loss of a functional group (R), the enzyme is 'aged' and initiates the steps leading to delayed neuropathy and less prominent acute toxic effects. By generating $Nte^{+/-}$ mice, Winrow *et al.*[7] showed that mice with less NTE and lower activity of NTE are more sensitive to the toxic effects of prototypical organophosphate compounds (*b*), findings that seem to rule out the toxic gain-of-function (aged) phenotype that serves as the key feature of the NTE hypothesis. Alternative possibilities should be considered (*c*) because organophosphates have the potential to phosphorylate a variety of key serine-containing substrates, leading to altered function of a given protein[10] and potentially accounting for acute and delayed neurotoxic effects.

inhibited NTE activity resulted in OPIDN. Only those organophosphates that both inhibited NTE by 70–90% and modified its structure to produce a negatively charged (aged) enzyme subsequently resulted in OPIDN[5]. Of course, there have been exceptions to this rule, but, by and large, aged NTE, like a fine wine, represents a product that has been widely appreciated. Although they are not the first to point out potential problems with the aging NTE concept, Winrow and colleagues[7] now report results that may damage this theory extensively.

They recognized that the multistep NTE hypothesis of OPIDN development might be an epiphenomenon, despite the elucidation of the structure of NTE and the assignment of some potential functions of NTE (in *Drosophila melanogaster*) that were linked to neurodegeneration[6]. They generated mice lacking NTE, which died during embryogenesis. Mice heterozygous with respect to disrupted *Nte* ($Nte^{+/-}$), however, survived and had lower levels of NTE protein and NTE activity but normal levels of acetylcholinesterase. Surprisingly, when the $Nte^{+/-}$ mice were exposed to a potent organophosphate inhibitor of NTE, ethyl octylphosphonofluoridate (EOPF), the toxic effects of this compound were enhanced (see figure). Thus, lower levels and activity of NTE—and not aging or, as Winrow *et al.*[7] so aptly describe it, a gain of function of NTE—seems to be responsible for the toxic effects of a prototypical organophosphate (EOPF) known to inhibit NTE and cause OPIDN.

Neurotoxic or not?

These findings refer to enhanced toxic effects (mainly mortality and motor activity changes) rather than specifically neurotoxic (neuropathological) effects; greater susceptibility of $Nte^{+/-}$ mice to OPIDN was not shown. Unfortunately, the greater sensitivity of the $Nte^{+/-}$ mice to the toxic effects of EOPF precluded the opportunity to increase the inhibition of NTE to the 70–90% level thought to be required for initiation of OPIDN, and the mice did not survive long enough to allow neuropathological examination for the presence of OPIDN-like effects. These are disappointing shortcomings of the study, but ones that potentially can be addressed in the future by using different, less acutely toxic organophosphate analogs.

Undoubtedly, some will criticize the results simply because they were obtained in a mouse model rather than the sensitive experimental species of choice, the chicken. Such criticisms seem shortsighted, however, as this particular mouse model of OPIDN[2] and others[8] show a delayed neuropathological profile consistent with known cellular targets of organophosphates seen in susceptible species. The findings of Winrow *et al.*[7] serve as a solid basis for future studies aimed at uncovering targets downstream of NTE, or in addition to NTE, that may be involved in OPIDN without the need to invoke hypothetical constructs, such as the gain-of-function aged enzyme.

In the rush to focus on NTE as a cause of OPIDN, the very real possibility that other phosphorylated proteins are important for development of acute and delayed effects of organophosphates (see figure) has been overlooked. Indeed, because protein phosphorylation represents the dominant mode of post-translational modification that underlies all neuronal function[9], it would be quite surprising for the toxic (acute or delayed) effects of organophosphates to be linked to organophosphorylation of only a few substrates. Candidates abound[10,11], and a few, such as key protein kinases (protein kinase A, calcium/calmodulin-dependent protein kinase II), components of the cytoskeleton and transcription factors[12,13], have recently been implicated in the development of OPIDN. As noted in previous commentaries[14,15], the neurotoxicology community would be well served by further application of molecular approaches to fundamental issues in the field. Redefining the relationship between NTE and the development of OPIDN serves as but one important example of the success of such endeavors. □

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Association between organophosphate exposure and hyperactivity?

To the editor:

Winrow and coworkers[1] have offered a potentially useful genetically modified mouse model for study of the health implications of altered expression of neuropathy target esterase (Nte). But their primary conclusion that "moderate reduction in Nte activity, by either reducing the amount of Nte protein through genetics or using a potent Nte inhibitor, leads to hyperactivity" is critically flawed. The key data justifying their conclusion, presented in Figure 6c and d, showed that wild-type ($Nte^{+/+}$) mice treated intraperitoneally with 1 mg ethyl octylphosphonofluoridate (EOPF) per kg body weight had a hyperactivity response equal to or greater than that observed in untreated genetically engineered $Nte^{+/-}$ mice with 40% lower intrinsic Nte enzymatic activity. Although it was quantified in untreated $Nte^{+/-}$ mice, Nte activity was not reported in the EOPF-treated mice. Evidence for Nte inhibition in EOPF-treated mice was only inferred by reference to results of an earlier study[2] in which intraperitoneal treatment with 5 mg of EOPF per kg body weight was described as inhibiting NTE activity in mouse brain by 85%. But in the same table that describes this response (Table 4 in ref. 2), intraperitoneal treatment with 1.3 mg EOPF per kg body weight is reported as causing no inhibition of Nte activity in mouse brain (only 1% difference from control). This dose of 1.3 mg per kg body weight is slightly higher than that used in the hyperactivity experiments described by Winrow *et al.*[1].

These data on EOPF and Nte inhibition suggest that activity of Nte in the brain was probably not reduced at the dose used in the experiments ascribing increased hyperactivity to Nte inhibition induced by EOPF treatment. In the absence of measurements of inhibition of Nte activity in the brain at a dose of 1 mg EOPF per kg body weight, and knowing that a dose of 1.3 mg EOPF per kg body weight did not inhibit activity of Nte in the brain in other similar experiments, the hypothesis that organophosphate-induced inhibition of Nte is causally linked to hyperactivity is not plausible.

James Bus[1], Jacques Maurissen[1], Brian Marable[1] & Joel Mattsson[2]

[1]The Dow Chemical Company, Midland, Michigan 48674, USA. [2]Dow AgroSciences, Indianapolis, Indiana 46268, USA.

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In reply

We appreciate the interest and enthusiasm resulting from our report describing the generation of *Nte*-haploinsufficient ($Nte^{+/-}$) mice. In their letter, Bus *et al.* conclude that "the hypothesis that organophosphate-induced inhibition of Nte is causally related to hyperactivity is not plausible". We disagree on the basis of four lines of evidence. First, they did not note that the earlier study used Swiss-Webster mice and the present study used 129S6/SvEvTac mice. We know that the activity of Nte in the brain is different in these two strains, and differences in detoxifying enzymes might also contribute to any apparent dose-response discrepancy.

Second, we show that $Nte^{+/-}$ mice have elevated motor activity relative to $Nte^{+/+}$ littermates. These mice are genetically identical except for *Nte* haploinsufficiency. The power of mouse genetics enables analysis of the effects resulting from manipulating a single gene through comparisons with a littermate that is otherwise genetically identical. As noted in their letter, the $Nte^{+/-}$ mice have a 40% reduction in Nte activity in brain homogenates. From these results, it can be concluded that a decrease in Nte levels and activity leads to an increase in motor activity. Third, when $Nte^{+/+}$ mice are exposed to 1 mg of EOPF per kg body weight, we again see an increase in motor activity similar to that seen with $Nte^{+/-}$ mice. EOPF has been shown to be a potent Nte inhibitor both *in vitro* and *in vivo* in Swiss-Webster mice and hens. Finally, $Nte^{+/-}$ mice are more sensitive than their $Nte^{+/+}$ littermates to the toxic effects of EOPF at 6–10 mg per kg body weight. The overlap between the genetic and EOPF exposure experiments leads directly to the hypothesis that inhibition of Nte, either chemically or genetically, can lead to hyperactivity.

Christopher J Winrow[1,3], John E Casida[2] & Carrolee Barlow[1,3]

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Organophosphate neurotoxicity: a new theory

Characterisation of *Nte*, the gene that encodes neuropathy target esterase (NTE), has opened up a new theory of how organophosphates, present in either pesticides or chemical-warfare agents, may cause delayed chronic neurotoxic syndromes. Previously, it was proposed that a gain in function of NTE was associated with greater organophosphate toxicity. But now Carrolee Barlow's group in California reveal that genetic or chemical reduction of *Nte* activity may be the main factor underlying the molecular mechanism of nerve damage.

Christopher Winrow (The Salk Institute for Biological Studies, La Jolla, CA, USA) and colleagues generated mutant mice and found that mice lacking NTE (*Nte*-/-) died during embryogenesis. Heterozygous mice (*Nte*+/-) survived but had lower concentrations of NTE protein and lower NTE activity, but normal levels of acetylcholinesterase, an enzyme that is inhibited in acute organophosphate toxicity. Surprisingly, *Nte*+/- mice were more susceptible to the effects of ethyl octylphosphonofluoridate, a potent organophosphate inhibitor of NTE (*Nat Genet* 2003; 33: 477–85).

"This turns our old theory of how organophosphates cause delayed neurotoxicity completely on its head", says James O'Callaghan, author of the accompanying editorial (*Nat Genet* 2003; 33: 437–38). Lower concentrations or lower activity of NTE, rather than a gain in function of NTE, lead to organophosphate toxicity through a mechanism that is not yet understood, he explains. Although Gulf War Syndrome is the most publicised example of delayed neurotoxicity caused by organophosphate exposure, the most well studied example is organophosphate-induced delayed neuropathy (OPIDN). "To date, about 30 000 human cases have been studied and the most common animal model of the disease is the hen", explains Barlow.

O'Callaghan observes that some will criticise the current results because they have not been obtained in the species of choice, but stresses that the mouse model provides a useful tool for future studies aimed at uncovering targets downstream of, or in addition to, NTE, that may be involved in OPIDN. "The use of *Nte*-deficient mice may also hasten the development of therapies for OPIDN and related conditions", comments Barlow. "It is always rewarding when experiments are done that allow us to look at a process in a new light. We hope these results and the mouse model can be used to help us better understand the disease physiology related to low and high dose organophosphate exposure and the contribution of NTE to diseases linked to exposure", she adds.

Kathryn Senior

Gulf War Syndrome Gene

03.25.03
email to a friend

As U.S. and allied soldiers in Iraq face the constant danger of chemical and biological weapons, scientists are still trying to figure out why some veterans of the last gulf war returned home with mysterious symptoms and brain damage.

This ScienCentral News video reports that neuroscientists are studying a gene that could make some people more susceptible to chemical agents.

Video

image: ABC News

(movie will open in a separate window)

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A Genetic Link

A new Gulf War means renewed concern about exposure to nerve gas, which is thought to contribute to Gulf War Syndrome, a loosely defined collection of symptoms, including chronic fatigue, diarrhea, migraines, dizziness, memory problems, loss of muscle control, and loss of balance.

Now scientists think they have found a genetic link between certain pesticides and chemical weaponry to a number of neurological disorders, including Gulf War Syndrome. During the Gulf War, thousands of soldiers could have been exposed to toxic nerve gases. At the same time, pesticides were widely used to ward off insect-borne diseases, a leading killer of servicemen in previous wars.

Some scientists used to attribute the symptoms to stress, because not all Gulf War veterans exhibited symptoms of the syndrome. “The cause of Gulf War Syndrome is a little bit controversial,” says Dr. Carrolee Barlow, professor at the Salk Institute for Biological Studies and molecular neuroscience director at the pharmaceutical company Merck. “It’s thought to be due to a combination of exposure to chemicals that are in some of the pesticides, or in some of the nerve gas that they could have been exposed to.”

Now there appears to be a genetic reason why some soldiers got sicker than others. The research team at the Salk Institute, headed by post-doctoral researcher Christopher Winrow, studied organophosphates, the toxic components of nerve agents and pesticides. They looked at the effects of these components on mice with either one, two or no copies of a gene called NTE.

Both mice and humans can have up to two copies of this gene, one from the mother and one from the father. The research team engineered mice to lack either one or both copies of the gene. The mice without the gene did not survive. The mice with only one copy of the gene survived, but reacted more strongly to the organophosphates than mice with two copies of the gene. “When we looked

Also on ScienCentral News:

Battlefield Band-Aids - Bleeding is still the number one cause of death on the battlefield. But that may change with the arrival of a new, high-tech bandage. (3/24/03)

High Tech Army Togs - Today’s soldiers are armed with so many high-tech gadgets that they’re advertised as "an army of one." Now it looks like one of those high-tech devices may be the uniform itself. (10/23/02)

Elsewhere on the web:

Gulf War Syndrome Looms Anew

Clinical Findings from Medical Examinations of U.S. Gulf War Veterans

Vaccines Linked to Gulf War Syndrome

at mice that had one copy of the gene disrupted and exposed those to the chemicals, we found that these mice were much more sensitive to the toxic effects of the chemical," says Winrow.

The NTE gene encodes for an enzyme called neuropathy target esterase, that can give nerve cells some protection against nerve agents. Researchers found that fewer copies of the gene led to less enzyme and more susceptibility to organophosphates. Over time this led to neurological problems, echoing symptoms similar to the Gulf War Syndrome. These findings, which will be published in the journal *Nature Genetics* on April 1st, are the first to demonstrate a clear genetic link between neurological disorders and exposure to organophosphate chemicals, which include household pesticides as well as nerve agents like sarin gas.

"What our study suggests is that if you inherit even one bad copy or one modified copy from either one of your parents, you'd be at increased risk if you are exposed to pesticides or nerve gas," says Barlow. People who have lower levels of NTE because of genetic variations might be at greater risk of damage from nerve gas toxins. They could be screened in advance, and take greater precautions.

Gulf War Syndrome was first noted after operations Desert Storm and Desert Shield in 1991. The Pentagon has identified about 130,000 troops it believes were exposed to low levels of sarin in 1991 when U.S. forces destroyed a weapons depot at Khamisiyah in southern Iraq. However some veterans, like Major Denise Nichols, a retired U.S. Air Force flight nurse who was stationed on the border of Iraq and Saudi Arabia during the last Gulf War, believe other nerve agent exposures occurred during that war.

Nichols, who suffers from Gulf War Syndrome, says that although she appreciates the efforts of scientists looking at the genetic aspect of the syndrome, the defense department should take more precautions to protect all soldiers against exposure to toxic chemicals. "Toxic overload to the body could be the reason. It's not one thing; it's the totality of all the things that the troops have experienced," she says. "We also need to look at how much the human body can take."

The research was supported by a grant from the U.S. Department of Defense.

top **email to a friend** **by Karen Lurie**

JAX® Mice Data Sheet

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Strain Name: 129S-*Nte*^{tm1Blw}/J
Stock Number: 005091

Price and Supply Information
General Terms and Conditions of Sale

Symbol(s) ***Nte*; *Nte*; *lacZ*; *lacZ*;**

Product Information
Strain Details

Type	JAX® GEMM® Strain - Targeted Mutation; Additional information on JAX® GEMM® Strains.
Investigator - Mutation Made By	Christopher J Winrow, Salk Institute for Biological Studies
Investigator - Donating	Carrolee Barlow, Salk Institute for Biological Studies
ES Cell Line	TC1

Strain Description
Homozygous null mice have an embryonic lethal phenotype, failing to develop past embryonic day 8.5. Mice that are heterozygous for the targeted mutation are viable, fertile, normal in size and are more active than wildtype littermates. A reduced level of protein gene product is detected by immunoprecipitation and Western blot analysis of brain, testes and kidney, but protein levels are not reduced in liver. Beta galactosidase activity in heterozygotes aged embryonic day 13.5 is found in the developing lens and spinal cord. In adult heterozygotes, beta galactosidase activity is detected throughout the brain, especially in the cerebellar Purkinje cells and hippocampus. Beta galactosidase activity patterns mimic the endogenous gene expression pattern. NTE activity in brain tissue is reduced by 40%. Heterozygotes are more sensitive to organophosphate toxicity with increased motor activity and mortality. This mutant mouse strain may be useful in studies of neuropathological hyperactivity, Gulf War Syndrome, and organophosphate-induced sub-acute neurotoxicity.

Strain Development
A targeting vector containing *lacZ*, neomycin resistance and herpes simplex virus thymidine kinase genes was used to disrupt exons 4 to 10. The *lacZ* coding sequence was inserted into exon 4, and exons 5 to 10 were deleted. The construct was electroporated

into 129S6/SvEvTac-derived TC1 embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6J blastocysts. The resulting chimeric animals from two independent ES cell clones were crossed. The offspring were mated to 129S1/SvImJ (STOCK#2448) mice.

Gene Details

Symbol *Nte*
Symbol Name neuropathy target esterase
Chromosome 8
Symbol Common Name(s) MSws; Swiss cheese;

Symbol *Nte*; *lacZ*
Symbol Name neuropathy target esterase; beta-galactosidase
Chromosome 8

Symbol *lacZ*
Symbol Name beta-galactosidase
Chromosome UN

Control Information

Control Notes Wildtype mice from the colony may be used as controls.

Genotyping Protocols

Nte tm1Blw

Colony Maintenance

Breeding and Husbandry This strain is maintained as a heterozygote due to homozygous embryonic lethality. Heterozygotes are more active than wildtype littermates.

Related Strains

004158	B6.129-*Maf*^{tm1Gsb}/J
005119	B6.129S6-*Npas2*^{tm1Slm}/J
003139	B6.Cg-Tg(DBHn-lacZ)8Rpk/J
003563	B6.Cg-Tg(tTALap)5Bjd/J
002982	B6.Cg-Tg(xstpx-lacZ)32And/J
005064	B6;129-*Slc30a3*^{tm1Rpa}/J
004849	B6;CBA-Tg(Tek-rTA,TRE-lacZ)1425Tpr/J

004141 B6;CBA-Tg(UAS-lacZ)65Rth/J
002369 B6;SJL-Tg(c177-lacZ)226Bri/J
002372 B6;SJL-Tg(c177-lacZ)227Bri/J
002621 B6;SJL-Tg(tetop-lacZ)2Mam/J
003299 B6;SWJ-Tg(TIMP3-lacZ)7Jeb/J
002865 B6CBA-Tg(Wnt1-lacZ)206Amc/J
002754 C57BL/6-Tg(LacZpl)60Vij/J
002193 C57BL/6J-Tg(MTn-lacZ)204Bri/J
002981 DBA/2-Tg(xstpx-lacZ)36And/J
003140 FVB/N-Tg(PAI1-lacZ)1Jjb/J
002856 FVB/N-Tg(TIE2-lacZ)182Sato/J
003315 FVB/N-Tg(tetORo1-lacZ)3Conk/J
003487 FVB/NJ-Tg(XGFAP-lacZ)3Mes/J
004623 STOCK Tg(Fos-lacZ)34Efu/J
002395 STOCK Tg(Zfy1-lacZ)218Bri/J
003274 STOCK Tg(tetNZL)2Bjd/J

Research Applications

This mouse can be used to support research in many areas including:

Neurobiology Research
Behavioral and Learning Defects(induced)

Research Tools
Neurobiology Research (genes regulating behavior and learning)
Toxicology Research

lacZ related

Research Tools
lacZ Expression

References

Primary Reference

Winrow CJ, Hemming ML, Allen DM, Quistad GB, Casida JE, Barlow C. 2003. Loss of neuropathy target esterase in mice links organophosphate exposure to hyperactivity. Nat Genet. 33 :477-85. [PubMed: 12640454]

Additional References

Price and Supply Information

This strain is currently Under Development for Distribution Colony.
To register your interest in this strain go to Strain Interest Form.

To View All Strains Under Development go to REGISTER INTEREST: New Strains Under Development.

Estimated Available for Sale Date: This strain is in our Importation facility. We do not yet have an

estimated available for sale date.
Please register interest in this strain by going to the Strain Interest Form. This will enable us to e-mail you availability information 3 weeks before the strain becomes available. We will post an estimated available for sale date as soon as possible.

Supply Details

Standard Supply	Level 10: Under Development for Distribution Colony.
Supply Notes	This strain is included in the Induced Mutant Resource collection.
Licensing	None. See **General Terms and Conditions of Sale** below.
Control Information	View Control Information in Strain Details. View Control Pricing Information for JAX® GEMM® Strains.

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The Jackson Laboratory has rigorous genetic quality control and mutant gene genotyping programs to ensure the genetic background of JAX® Mice strains as well as the genotypes of strains with identified molecular mutations. JAX® Mice strains are only made available to researchers after meeting our standards. However, the phenotype of each strain may not be fully characterized and/or captured in the strain data sheets. **Therefore, we cannot guarantee a strain's phenotype will meet all expectations.** To ensure that JAX® Mice will meet the needs of individual research projects or when requesting a strain that is new to your research, we suggest ordering and performing tests on a small number of mice to determine suitability for your particular project.

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The Jackson Laboratory

Nte ^{tm1Blw}, Version 1

strains -------- 005091

Created on: 22-sep-2004 14:52:49 Updated on: 22-sep-2004 14:52:49

Notes: *Use 2µl (5-20ng) DNA per reaction. DNA loading dye = 60% sucrose/5mM cresol red. Our genotyping laboratory purchases Cresol Red (Sodium Salt) from Sigma, catalog number C9877.

Products **Gel Image**

+/+ = 350bp
+/- = 350/700bp

-/- = 700bp

Reaction Components

			# of Rxns:	1	Calculate
Reaction Components	**Vol/Rxn A**	**Vol/Rxn B**	**Final Conc**	**Total Volume A**	**Total Volume B**
H20	3.280	0.000	QS to 12 µl	3.28	0.00
10X PE Buffer II	1.680	0.000	1.4X	1.68	0.00
25 mM MgCl2	0.960	0.000	2 mM	0.96	0.00
2.5 mM dNTP	0.960	0.000	0.2 mM	0.96	0.00
20 µM oIMR 3531	0.600	0.000	1.0 µM	0.60	0.00
20 µM oIMR 3532	0.600	0.000	1.0 µM	0.60	0.00
20 µM oIMR3533	0.200	0.000	0.33 µM	0.20	0.00
DNA dye	1.660	0.000	0.138 µl/µl TV	1.66	0.00
5 U/µl Taq Pol.	0.060	0.000	0.025 U/µl	0.06	0.00
DNA (2 µl per Rxn)**	2.000	0.000	(2 µl per Rxn)**	2.00	0.00

Cycling Conditions

Cycling Reaction A	Step	Temp	Time	Note
	1	94 °C	3 min	
	2	94 °C	30 sec	
	3	65 °C	1 min	
	4	72 °C	2 min	Go to step 2, 35 times
	8	72 °C	2 min	
	9	10 °C		

Separated by gel electrophoresis on a 1.5% agarose gel.

Primers

oIMR3531	5'- 5'-CTT CCg CAT AAT CTT CCg gCC A-3' -3' Tm = °C	22-mer A=4, C=9, G=3, T=6
oIMR3532	5'- 5'-TgT gCC CgT TCg	

	TTC CgC Ag-3' -3' Tm = °C	20-mer A=1, C=7, G=6, T=6	Primer NTE2.23 from the DI. It is suspected that this is the common primer.
oIMR3533	5'- 5'-TgA TCT TCC AgA TAA CTg CCg TCA CTC C-3' -3' Tm = °C	27-mer A=6, C=9, G=4, T=8	Primer B-GalREvNT from the DI. Note: this strain is homo lethal. The DI recommends 60C for 1 min. The wt product is 350bp and the mutant product is 700bp. 9-3-04 TB.

This genotyping protocol is used for the following strains:

Stock Number	Strain Name
005091	129S-*Nte*^{tm1Blw}/J

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